The biophysics and biochemistry of a cochlea-like organ in the ear of Neotropical bush-crickets (Insecta: Tettigonidae)

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Declaration

I declare that no material contained in the thesis has been used in any other submission for an academic award at this or any other institution. I declare that the thesis is all my own original work, except where otherwise indicated.

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Abstract

There has been an increasing interest in the study of complex auditory processes in the mammalian cochlea (e.g. frequency resolution, frequency discrimination and active amplification). These processes depend on the propagation of frequency information in the form of travelling waves (of the type exemplified in a tsunami) along the tonotopically arranged auditory sensilla. The physiological and biophysical bases of traveling waves in the mammalian cochlea remain elusive, yet vital to understanding tonotopy (the mapping of sound frequency across space) and active amplification. In vertebrates, both location and osseous protective material make the inner ear difficult to access without altering its integrity. While conventional methods for hearing research in vertebrates have improved notably in recent years, these still require surgical procedures to gain physical access to the inner ear, compromising the natural conditions of the hearing system. Indeed, measurement of auditory activity *in-vivo* has only been done through small surgical openings or other isolated places. Remarkably, complex auditory processes are not unique to vertebrates, and similar mechanisms for sound filtering, amplification, and frequency analysis have also been found in the ears of insects. Hearing organs in insects are unusually small, highly sensitive, and easily accessible by means of non-destructive methods. Among insects, bush-crickets (Insecta: Orthoptera) have a unique hearing system which consists of minute tympanal ears located in the forelegs, and inner ears with tonotopically organised auditory sensilla within a fluid-filled cavity. Unlike in vertebrates, the bush-cricket inner ear is not coiled, but stretched. Critically, the assessment of auditory processes in this small-scale ear is proposed to be possible in a non-

invasive manner. The purpose of this thesis was to further the knowledge of acoustic perception in bush-crickets by providing new data on the travelling wave phenomenon, the suitability of bush-crickets for non-invasive experimentation, and the elemental composition of the liquid contained in the bush-cricket inner ear. It was demonstrated that transparency is the cuticle property that allows the observation and measurement of travelling waves and tonotopy in bush-crickets through the use of light measurement techniques, specifically laser Doppler vibrometry. This approach provides a non-invasive alternative for measuring the natural motion of the sensillia-bearing surface embedded in the intact inner ear's fluid. Subsequently, this experimental technique was used to generate novel data on inner ear mechanics from a number of bush-cricket species. Finally, in the form of a chemical analysis, I established that the inner ear's liquid differs from the hemolymph based on the variation of their ion concentration values. From a biomechanical perspective, the presence of a liquid-filled cavity along with a species-specific ion concentration, likely contributes to an optimal functioning of the hearing organ just as it occurs in vertebrates. These results highlight the importance of considering analogous models of vertebrate hearing systems for advanced studies of auditory function. Such models can be used to effectively observe, collect, and measure auditory data otherwise impossible to attain non-invasively in vertebrates, and specifically mammalian species.

Preface

All authors of the following publications have contributed in various degrees to the research concepts, the experimental designs and the manuscript of this thesis.

Journal Publications

Sarria-S, F. A., Chivers, B. D., Soulsbury, C. D., & Montealegre-Z, F. (2017). Non-invasive biophysical measurement of travelling waves in the insect inner ear. Royal Society Open Science, 4(5), 170171.

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Conference talks

Sarria-S F. A., & Montealegre-Z F. (2016). Let me see what you hear: transparency in katydid auditory systems. 12th International Congress of Orthopterology.

Sarria-S, F. A., Gonzalez-R, J., & Montealegre-Z, F. (2016). Transparency in bush-cricket auditory systems (2016). PGR Symposium, University of Lincoln, UK

Sarria-S, F. A., Jonsson, T., & Montealegre-Z, F. (2015). Non-invasive measurement of travelling waves in the inner ear of neotropical bushcrickets. XXV meeting of the International Bioacoustics Council (IBAC).

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Chapter 1: Introduction

1.1. Animal communication

In the animal kingdom, each single living system (from microorganisms to vertebrates) is in constant exposure to its surrounding physical and biological environment. In order to cope with survival and being able to reproduce, animals possess highly developed sensory systems capable of detecting and responding to static and dynamic cues from their environment (Barth et al., 2012; Endler, 1993; Manley, 2012). Under this context, the perception of random environmental cues has evolved into more complex mechanism of signalling between individuals of the same species. The main effect of this process is the occurrence of sensory organs with high sensitivity for specific stimuli and efficient signal amplification (Endler, 1992; Frings, 2012). Animal communication can be classified in three basic categories according to the type of sensory receptor involve in the process: Chemoreceptors, photoreceptors, and mechanoreceptors (Ryan, 1990).

1.1.1. Chemical communication

Chemical signals are exploited by all animal groups and play a crucial part in mate attraction, predation, and social interaction (Wilson, 1970). Chemical compounds secreted or excreted by an individual to the environment, and capable of affecting the behaviour of a conspecific receiver, are known as pheromones (Alcock, 1989; Karlson and Lüscher, 1959; Wyatt, 2003). These type of signals can be transmitted over long distances, in the case of small organism these long distances represent millimetres and kilometres for large animals (Penn and Potts, 1998). Their transmission is not affected by the

presence or absence of light, it can circumvent physical barriers present in the environment (e.g. trees, foliage, rocks, etc.), and their synthesis requires minimum metabolic investment (Table 1.1.). Pheromones can also last for days and be transferred directly from signaller to receiver. Depending on their function, pheromones are divided in three categories: Sex, aggregation, and alarm pheromones (Wilson, 1970). Sex pheromones are important in many taxa for species recognition, sex identification and assessment of the reproductive state of potential mates (Eisner and Meinwald, 1995; Landolt, 1997). In contrast to sex pheromones, aggregation pheromones act in both sexes, including immature stages. Aggregation pheromones recruit group members to new sources of food, to defend the territory, or to protect the group against enemies (Wyatt, 2003). In social insects alarm pheromones are the most commonly produced class of chemical signal, after sex pheromones, and have evolved independently within all major taxa (Blum, 1969).

Although, pheromones transfer information over distance, one of the disadvantages of this mode of communication is the slow transmission process and low information exchange rate (Bradbury and Vehrencamp, 1998).

1.1.2. Visual communication

As in chemical communication, visual signals are common among all animals, but only three taxonomic Phyla (Mollusca, Arthropoda, and Chordata) have evolved optical organs for processing visual stimulus like colour, shape and motion (Greenfield, 2002; Ryan, 1990). In this mode of communication, signals

are transmitted as electromagnetic waves that travel over long distances at high velocities (speed of light: approximately $3.00x10^8$ m/s), with features such as frequency, duration, and direction providing information content. In fireflies, for example, pulses of light with a wavelength between 560 and 630 nm are used in courtship interactions between males and females, and each species has its unique display pattern and response time (Carlson and Copeland, 1985; Demary et al., 2006; Lloyd, 1966; Wilson and Hastings, 1998). Unlike pheromones, optical signals require a clear line of sight, are dependent of daylight (Except for animals using bioluminescence). Additionally, visual displays like body movements and bioluminescent bursts require a high metabolic investment (Woods Jr et al., 2007).

1.1.3. Mechanical communication

This type of communication includes acoustic and vibrational signals. These signals can be transmitted through a fluid medium or solid substrate (Mason and Pollack, 2016). They are independent of light and move through the space with minor restrictions (Greenfield, 2002; Hoy and Fay, 2012). Their physical properties such as frequency, amplitude, and directionality provide a diverse and complex range of information (Fletcher, 1992; Marten et al., 1977). For example, signals might convey a broad message, such as species identity between groups, or a narrower message, such as information about the identity of a specific individual within a group (Simmons et al., 2003). Another advantage is that this type of signal can be rapidly started, stopped, or modified to send a time-sensitive message in which a single (seismic communication) or multiple recipients (acoustic signals) are involved.

In terms of sound production, acoustic signals can be generated by two mechanism: vocalization and stridulation. Vocalization is predominantly used by vertebrates and involves the conversion of an air flow, passing through an oscillating structure, into acoustic energy (Fitch and Hauser, 2003; Simmons et al., 2003). Whereas stridulation is the muscle-driven mechanical interaction of two body parts and it is the main mechanism adopted by arthropods (Dumortier, 1963; Ewing, 1989), among them crustacean (Patek, 2002; Schmitz, 2002) and insects (Greenfield, 2002; Michelsen and Nocke, 1974). Other mechanisms for sound production include the expulsion of air through the spiracles, as occurs in the hissing cockroach *Gromphadorhina portentosa* (Nelson and Fraser, 1980), and the tymbal organs of cicadas, a pair of sclerotized dome-like membranes (Bennet-Clark, 1999; Pringle, 1954). These membranes vibrate rapidly producing a “clicking” sound that is amplified by a plate on the membranes (Young and Bennet-Clark, 1995).

As in other arthropods, acoustic communication has been developed in insects thanks to the plasticity of the exoskeleton for the adjustment of any body part into sound production structures (Bennet-Clark, 1975; Bennet-Clark, 1999; Elsner and Wasser, 1995). Among insects, this property has influenced the evolution of a diversity of long distance acoustic communication systems (Bailey, 1991; Ewing, 1989; Greenfield, 2002; Michelsen and Nocke, 1974; Robinson and Hall, 2002; Yager, 1999). For instance, in cicadas (Homoptera: Cicadidae), males produce loud sounds through a modified region of the exoskeleton that forms a complex membrane with thin, membranous portions and thickened ribs (Bennet-Clark and Young, 1992; Fonseca and Bennet-

Clark, 1998; Pringle, 1954; Young and Bennet-Clark, 1995; Young and Josephson, 1983). In Orthoptera, acoustic signals are generated by mechanical friction. This form involves body segments or appendages that are rubbed together and regardless of their anatomical position, they have two common structures: a file and a plectrum (Michelsen and Nocke, 1974). Furthermore, tegmino-tegminal stridulation is observed in Gryllidae, Gryllotalpidae, and Tettigoniidae of the suborder Ensifera (Bailey, 1970; Montealegre-Z et al., 2011; Montealegre-Z and Mason, 2005), while femur-wing interaction occurs in Acrididae of the suborder Caelifera (Robinson and Hall, 2002; von Helversen and von Helversen, 1997). In Diptera, sound production involves wingbeat movements as seen in the families Drosophillidae, Tephritidae, and Cullicidae (Bennet-Clark, 1971; Bennet-Clark et al., 1980; Jackson and Robert, 2006; Sivinski and Webb, 1985). Unlike Orthoptera and Hemiptera, the acoustic signals produced in Diptera are low in frequency and are effective over relatively short distances (Cator and Zanti, 2016; Robert, 2009). Although, a good number of species from different insect groups generate some sort of sound, acoustic communication is seen to be predominant in Orthoptera (Ensifera and Caelifera) and in Hemiptera (Cicadidae and Corixidae), with a few exceptions (Greenfield, 2016).

Table 1.1. Summary of main features of the described sensory channels of communication.

	Type of signal			
Feature of channel	Chemical	Acoustic	Visual	Mechanical
Range	Long	Long	Medium	Short
Transmission rate/Flow round barrier	Slow/Yes	Fast/Yes	Fast/No	Fast/No
Locatability of sender	Difficult	Medium	High	High
Energetic cost to sender	Low	High	Moderate	Low
Duration	Variable	Instantaneous	Moderate	Short
Use in darkness	Yes	Yes	No, unless bioluminescence is used	Yes
specificity	High	High	Medium	Limited

After (Alcock, 1989).

1.2. Acoustic perception

Sensory structures sensitive to acoustic signals in air or water have been recognised in vertebrates, some arthropods and cephalopods. In vertebrates, auditory structures are found in all classes (Greenfield, 2016), while in arthropods and cephalopods, hearing organs are present only in few taxa. In insects, pressure-sensitive ears are found in 9 orders (Göpfert and Hennig, 2016; Hoy and Fay, 2012), with Orthoptera and Hemiptera being the two orders in which sound is the predominant communication system. In arachnids and crustaceans recent studies have shown that some species respond to sound pressure waves (Gordon and Uetz, 2012; Hughes et al., 2014; Shamble et al., 2016). Equally, in some cephalopods, sensitivity to waterborne sound has been reported (André et al., 2016; Hu et al., 2009; Mooney et al., 2010),

yet there is still lack of evidence of the use of acoustic signals for intraspecific communication (Kaifu et al., 2011).

Vertebrate hearing systems are structurally diverse, however, despite the differences in anatomy, they have evolved analogous mechanisms for sound detection and analysis (Christensen-Dalsgaard and Carr, 2008; Fay and Popper, 2000; Manley, 2012). Furthermore, another common feature is the active filtering and active amplification of distinct spectro-temporal features of sound. This feature increases the range of detection and discrimination of relevant acoustic signals in a noisy environment (Albert and Kozlov, 2016; Fay et al., 1992; McGregor, 2005).

Frequency analysis in the vertebrate ear depends on mechanical and electromechanical properties of the inner-ear, but in mammals, the propagation of frequency information is delivered in the form of a travelling wave along a tonotopically arranged auditory sensillia (Robles and Ruggero, 2001; von Békésy, 1960). The physiological and biophysical basis of travelling waves in the inner-ear remains elusive (Montealegre-Z and Robert, 2015), yet vital to understanding tonotopy (mapping of sound frequency across space) and active amplification (Mhatre, 2015).

In the case of insects, environmental and ecological factors have shaped the form and function of hearing organs over evolutionary time (Forrest, 1994; Yager, 1999). Hearing organs have evolved independently in at least 9 taxa (Göpfert and Hennig, 2016) and they can be found in different places of the

insect body, including mouthparts, antennae, wings, or legs (Hoy and Robert, 1996; Strauß and Lakes-Harlan, 2014; Yack, 2004). Most of the auditory organs in insects are derived from chordotonal organs (Field and Matheson, 1998; Yack, 2004) and depending on the sound-receiving morphological structures, insect ears can be classified into antennal or tympanal ears (Hoy and Fay, 2012; Michelsen, 1979; Robert, 2004). Antennal ears respond to the particle velocity component of sound, which sets the antenna into vibration (Jackson and Robert, 2006). On the other hand, tympanal ears have a sound-receiving eardrum backed by an air-filled space and vibrates in response to the pressure component of sound (Hoy and Robert, 1996; Jonsson et al., 2016; Michelsen and Larsen, 2007).

Despite the clear differences in anatomy and the evolutionary separation between vertebrates and insects, the physical principles underlying sound production and detection determine the functioning of the hearing system in both taxonomic groups. Research on the antennal ear of Drosophila fruit flies (Senthilan et al., 2012) has suggested at the molecular level that vertebrate hair cells and insects chordotonal neurons might be evolutionary related (Pierce et al., 2008). Likewise, detailed analyses of the mechanics of insect tympanal organs have identified other shared features with vertebrate systems, including traveling waves (Malkin et al., 2014; Palghat Udayashankar et al., 2012; Stephen and Bennet-Clark, 1982; Sueur et al., 2006; Windmill et al., 2005), cochlea-like mechanics in bush-cricket ears (Montealegre-Z et al., 2012), and processes of active amplification (Göpfert and Robert, 2008; Mhatre, 2015; Mhatre and Robert, 2013). These analogies represent an

opportunity to further the study of acoustic perception in non-mammalian systems.

1.3. Model of study

Bush-crickets, taxonomically, are classified in the Tettigoniidae family (order: Orthoptera) in which approximately 7104 species have been described (Song et al., 2015). These insects have a wide geographical distribution (Bailey and Rentz, 1990; Gwynne, 2001) with a majority of species found in the tropics (Cigliano et al., 2017; Heller, 1995). Compared to other insects, some species of tropical bush-crickets are of a considerable size (2-8 cm), which has enabled in depth studies of their ears and signal processing at neurological levels (Robinson and Hall, 2002). Research in sound production and perception in Orthoptera insects has inspired valuable information that has helped to understand complex interspecific and intraspecific ecological aspects such as predation, parasitism and competition (Robinson and Hall, 2002; Römer et al., 2010), as well as to demonstrate the response to environmental physical phenomena through specific anatomical and behavioural adaptations (Heller and von Helversen, 1993; Montealegre et al., 2014; Römer, 1993; Schul et al., 2000; Siegert et al., 2013; Simmons and Zuk, 1992; von Helversen and von Helversen, 1995). Consequently, from an experimental viewpoint, orthopterans represent an attractive alternative for the study of acoustic perception and its associated behaviour in land-dwelling animals.

1.3.1. Sound production and hearing in Tettigoniidae

Tettigoniidae, as other ensiferans in the order Orthoptera, are insects that exploit acoustic signals to interact with their conspecifics, prey detection or predator avoidance (Gerhardt and Huber, 2002; Gwynne, 2001; Simmons, 1988). Mostly, adult males produce mating calls by tegminal stridulation to attract distant females (Fullard and Yack, 1993; Montealegre-Z et al., 2009; Morris, 1999). In brief, the sound is produced by the mechanical interaction of the forewings (Figure 1.1). One of the wings (Generally the right tegmen) accommodates a scraper and sound radiating cells (mirror and harp), while the other wing on its ventral surface has a modified vein with a series of teeth resembling a file (Bailey, 1970; Michelsen and Nocke, 1974; Montealegre-Z and Mason, 2005; Montealegre-Z et al., 2009). The generated calling song is species-specific with respect to its frequency, temporal pattern, and repertoire (Fletcher, 1992; Göpfert and Hennig, 2016; Heller and von Helversen, 1993). These acoustic features allow females to recognize conspecific males and trigger a positive phonotatic response (Alexander, 1967; Morris et al., 1978; Schatral and Bailey, 1991; Strauß and Lakes-Harlan, 2014; Stumpner and Nowotny, 2014).

Figure 1.1. Bush-cricket stridulatory apparatus. A) Dorsal view of wings and stridulatory structures location in *Acanthodis* sp. Inset: B) Close up of the structures involve in the sound production: Mirror, plectrum and file. C) Ventral view of the file.

Acoustic signals are perceived by both males and females through paired tympanal organs located on their fore legs (Figure 1.2), just below the femoro-tibial joint (Bailey and Rentz, 1990; Hill and Oldfield, 1981; Hoy and Robert, 1996; Yack, 2004). These type of ears are characterized by a pair of tympanal membranes or ear drums which vibrate in response to airborne sounds. On most bush-cricket tympana, two topographical areas can be distinguished: the tympanal membrane and tympanal plate (Schumacher, 1975) (Figure 1.3). The former is larger and internally is backed by a tracheal air space, while the latter is smaller, more sclerotized and backed by a fluid filled cavity. At the interior of the fluid filled cavity is the auditory sensory organ. This consists of scolopidial sensory units which are indirectly activated by sound induced by tympanal oscillations (Boyan, 1993; Field and Matheson, 1998; Hoy and

Robert, 1996; Lakes and Schikorski, 1990; Strauß and Lakes-Harlan, 2014; Yack, 1992; Yack, 2004).

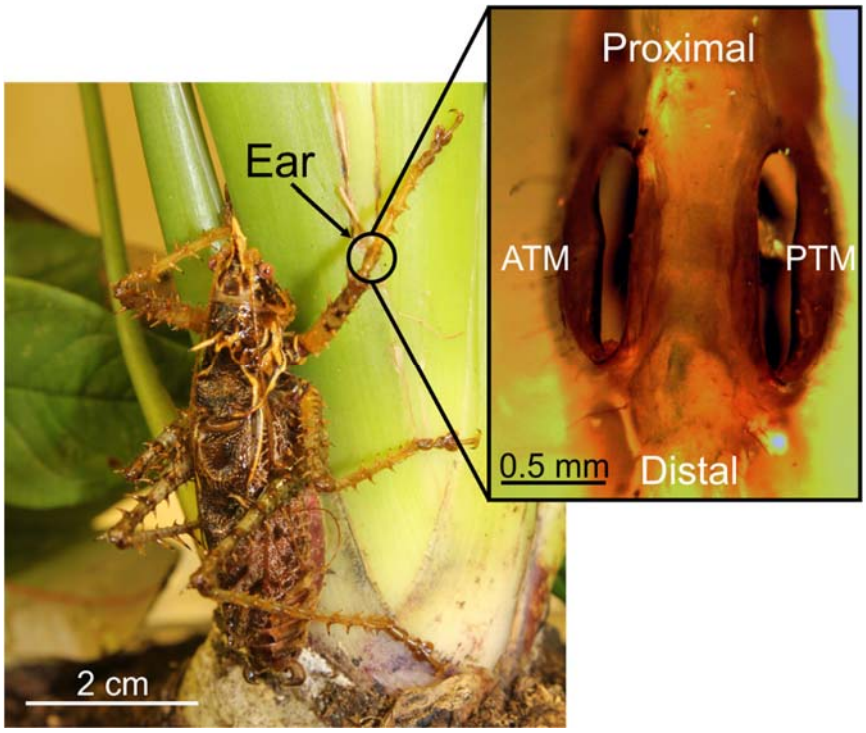

Figure 1.2. Morphology of the ear in the foreleg of *Panacanthus lacrimans*. The inset shows a close-up view the tympanal organ: anterior tympanal membrane (ATM), and posterior tympanal membrane (PTM).

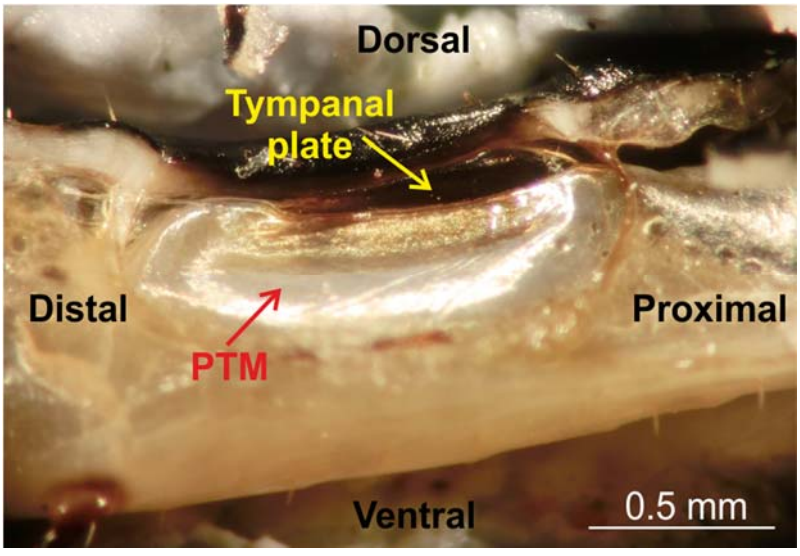

Figure 1.3. Lateral view of an exposed posterior tympanal membrane (PTM) and tympanal plate in the left foreleg of *Nastonotus foreli*.

1.3.2. Crista acustica and auditory vesicle liquid

Bush-cricket hearing organ derives from a set of stretch receptors known as chordotonal organs (Strauß and Lakes-Harlan, 2009). Like other chordotonal organs, the auditory receptor or *crista acustica* (Bangert et al., 1998; Schumacher, 1975) is composed of multicellular units called scolopidia and they are fixed to the acoustic trachea by one or more attachment cells (Oldfield, 1982; Yager, 1999). The scolopidia are arranged by size, with the smallest taking place at the distal end of the tibia (Rössler, 1992; Schumacher, 1973). In addition, the CA is not attached directly to the tympanal membranes, but instead lies directly beneath the leg cuticle (Figure 1.4).

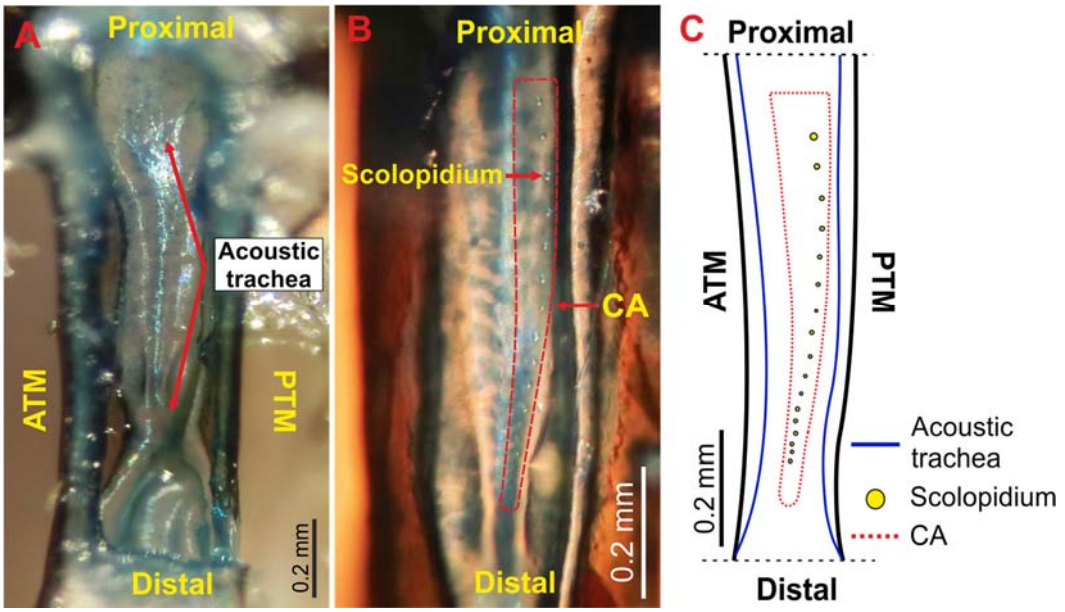

Figure 1.4. Dorsal view of the exposed tympanal organ in the foreleg of *Nastonotus foreli*. A) Acoustic trachea, anterior tympanal membrane (ATM), posterior tympanal membrane (PTM). B) Close up view of the crista acustica (CA) showing the scolopidia arranged in a row. Note the gradual increase in size from the tibia distal to proximal region. C) Schematic representation of the tympanal organ (Acoustic trachea, crista acustica, and scolopidia).

As in most bush-crickets, the haemolymph channel in the forelegs runs along of the dorsal surface of the acoustic trachea (Kalmring et al., 1994), and previous morphological descriptions of the auditory organ considered this to be covered by the insect's blood (Oldfield, 1985). Based on anatomical studies of the hearing system of the Neotropical species *Copiphora gorgonensis*, Montealegre-Z et al. (2012) observed that the hemolymph channel in this species was not continuous as it has been suggested before, but instead, they found a fluid trapped in a cavity bounded by the presence of a colloidal material at each end of the CA. Apolar extraction suggested that the chemical composition of the fluid has a lipid nature (Montealegre-Z et al., 2012). This cavity was denominated the auditory vesicle (AV) and it is considered to be an important element of the auditory system as it provides a medium for wave propagation and influences the dynamics of the tympanal membranes (Montealegre-Z and Robert, 2015). A fluid filled cavity appears to be a required condition for the function of auditory organs in bush-crickets and some other orthopterans, for instance, in the tree weta *Hemideina thoracica*, the auditory sensory organ is immersed in a fluid-filled channel composed primarily of a new class of fatty acid (Lomas et al., 2012).

The fluid-filled inner ear in bush-crickets resembles the mammalian cochlea with its three fluid-filled chambers: scala tympani, scala media, and scala vestibuli (Wangemann and Schacht, 1996). The AV and its liquid is mechanically comparable with the perilymphatic duct where the liquid medium enhances the propagation of TW. From the mechanical viewpoint, the Inner ear liquid together with the CA constitutes a spring-mass-damper system

(Babbs, 2011). This arrangement might provide the unique conditions for travelling wave propagation and frequency analysis by tonotopy in the bush-cricket inner ear.

In mammals, the cochlear fluids have a crucial role in the hearing process, they provide a dispersive medium (a material in which waves of different frequencies travel at different speeds) and a specific ionic environment for the functioning of the sensory cells. Unlike other insects, the bush-cricket auditory organ is immersed in a liquid medium that, according to preliminary studies (Montealegre-Z et al., 2012), appears to have a unique chemical composition. Therefore, in order to establish the relationship between the liquid contained in the AV and the bush-cricket auditory response, part of this thesis is intended to extend the available information of the chemical composition of the Inner ear liquid and provide a preliminary description of its ionic profile.

1.3.3. Travelling waves in the ear of Bush-crickets

Bush-crickets exhibit a sophisticated hearing system that includes an outer, middle, and an inner ear, which is functionally analogous to the mammalian system. During the hearing process in mammals, airborne sound waves are transformed into a mechanical force that acts on the ciliated structure of the sensory neuron (Fettiplace and Hackney, 2006). This process is influenced by the anisotropic properties of the basilar membrane (stiffness gradient and surface area) and the sound-induced travelling waves (Robles and Ruggero, 2001).

An analogous morphology-based tonotopy has also been described for the Tettigoniidae auditory system (Hummel et al., 2017; Kalmring et al., 1993; Lin et al., 1993; Oldfield, 1982; Römer, 1983; Rossler and Kalmring, 1994; Stolting and Stumpner, 1998). Similar to the mammalian cochlea, sound-induced travelling waves originate at the narrow distal, high-frequency end of the auditory organ, and propagate towards the wide low-frequency, proximal region of the same structure (Montealegre-Z et al., 2012; Palghat Udayashankar et al., 2012). This mechanical motion enhances the tonotopic response at a specific resonant location where the travelling waves reaches its maximum displacement (Hummel et al., 2017).

1.4. Research scope

As it has been mentioned, acoustic communication is widespread in vertebrates and in some invertebrates. In vertebrates there is a diversity of morphological adaptations for sound production and perception. Equally, insects under the same environmental and ecological pressure have developed specialised organs to generate and perceive acoustic signals. Research regarding the hearing process in vertebrates and insects has resulted in a comprehensive understanding of how auditory organs perceive, analyse, and convert mechanical stimuli into electro-chemical signals. Although, there is a considerable amount of data concerning the hearing process, there is still the need for more detailed studies regarding mechanical and chemical processes of some structural elements (i.e. sensory neurons, accessory membranes, or liquid environment). Equipment and experimental techniques have been a drawback in the study of auditory mechanisms in

vertebrates and insects, yet the main disadvantage has being the difficulty of accessing, in a non-invasive manner, the auditory organs. Recent experimental methods have allowed to overcome this by the combination of non-contact measurement techniques and species that required minimal to no manipulation of the hearing organ (Sarria-S et al., 2017).

Among insects, bush-crickets represent the most sophisticated auditory system, with elements and processes analogous to those observed in vertebrates. Furthermore, bush-crickets, much like other insects and vertebrates, have been exposed to environmental and ecological pressures that have influenced the development of ears capable of detecting and analysing the frequency component of a broad range of acoustic signals. Under this perspective, bush-crickets represents an alternative system to vertebrate models (i.e. chinchilla or guinea pig) to further the study of hearing processes. Despite the recent advances in insect hearing research, and in particular in bush-crickets, (Göpfert and Hennig, 2016; Montealegre-Z and Robert, 2015), there is still the need to address some features of the hearing process. So far there is an agreement regarding the contribution of travelling waves to the ear's tonotopic response, but the mechanisms involved in the transformation of mechanical motion into neural signals are still elusive. Hummel et al. (2017) have provided a preliminary approach to this, but the experimental technique remains invasive (cuticle ablation and ear liquid drainage). Additionally, the liquid bathing the sensory organ still requires in depth studies to elucidate its chemical and mechanical properties. Therefore, the purpose of this research is to contribute to the knowledge of the acoustic

perception in bush-crickets with a non-invasive approach and with analytical chemistry techniques to provide new data on the travelling wave phenomenon, the suitability of bush-crickets for non-invasive experimentation, and a preliminary description of the ionic composition of the liquid contained in the bush-cricket inner ear. In order to achieve this, the project was developed in three main experimental steps which are described as follow.

The cuticle protecting the sensory organ is the principal anatomical feature that limits the study of auditory perception in intact bush-crickets. Thus, in chapter 2, cuticle transparency is addressed as the main factor allowing the detection of auditory activity by using laser Doppler vibrometry. Cuticle transparency was quantified by measuring the transmittance (ratio of the transmitted radiant flux to the incident radiant flux) of cuticle samples covering the hearing organ. To test the effects of the cuticle transparency and thickness on the laser measurements, an experimental set-up was developed. The experiment involved the use of cuticle samples from the dorsal ear area and a reference vibratory surface. Once, the transmittance values were obtained, the next step was to corroborate the effect of cuticle transparency on laser Doppler vibrometry measurements *in-vivo*, presented in chapter 3. For this part of the project, specimens of *Phlugis poecila* were used since this species presented the highest transmittance values. The recorded auditory activity was recognised as a frequency spatial dependent response and a mechanical motion identified as a travelling wave was also recorded. In chapter 4, the elemental composition of the auditory vesicle liquid and a description of the used chemical analysis is reported. Finally, in chapter 5, I focus on a

discussion of the relationship of the physical and chemical properties studied in this project and the *in-vivo* measurements of auditory activity such as travelling waves and frequency tuning in intact bush-crickets.

Chapter 2: Cuticle transparency

Parts of this chapter have been published in:

Sarria-S, F. A., Chivers, B. D., Soulsbury, C. D., & Montealegre-Z, F. (2017). Non-invasive biophysical measurement of travelling waves in the insect inner ear. Royal Society Open Science, 4(5), 170171.

Contributions: My participation on this paper consisted in the realization of field work for specimen's collection, designing and running laboratory experiments, data collection and analysis, and writing and editing the manuscript.

It is hypothesised that cuticle transparency is the main factor allowing the measurement of traveling waves and spatial mapping of frequencies on the tympanal ear of bush-crickets. In this part of the project, cuticle transparency was quantified across six species and the correlation between this property, cuticle thickness and laser Doppler vibrometry measurements of bush-cricket auditory activity was established.

2.1. Introduction

In vertebrates the coiled shape and osseous protective material make the inner ear difficult to access without altering its integrity (Palghat Udayashankar et al., 2012; Robles and Ruggero, 2001; Young, 2007). Measurements *in vivo* have only been done through small openings in the scala tympani or other isolated places (Russell and Nilsen, 1997a; Young, 2007). Recent, less invasive approaches (e.g. optical coherence tomography) still require dissection of the skull to access the cochlea (Lee et al., 2015). Indirectly, the spatial frequency response on the basilar membrane (BM) has also been inferred through computational models (Mammano and Nobili, 1993; Mammano, 1990; Nobili et al., 2003), or estimated from auditory afferent nerve

fibres at selected points (Elliott and Shera, 2012; Lagarde et al., 2008). Hitherto, there lacks an easy, non-invasive approach to access the complex auditory processes occurring within the cochlea.

Unlike mammals, the bush-cricket “cochlea-like” organ is naturally uncoiled and the location in the forelegs makes them remarkably accessible (Figure 2.2). Using laser Doppler vibrometry (LDV), Montealegre-Z et al. (2012) measured travelling waves and frequency mapping in intact ears of the bush-cricket *Copiphora gorgonensis* (Figure 2.2). The authors reported that the auditory activity was recorded through the dorsal leg cuticle covering the ear, and no further manipulation of the system was required. In addition, Montealegre-Z & Robert (2015) stated that the same LDV protocol was implemented on other species, but it was observed that TW and tonotopy measurements could only be achieved in a small number of species tested. Based on this, they speculated that in those species on which recording of the inner processes were achieved, the exoskeleton surrounding the ear had a certain level of translucency, allowing the laser beam to be focused directly in the CA. However, this has neither been demonstrated nor measured.

Insects’ cuticle coloration is derived from the occurrence of a diversity of molecular pigments in combination with structural components. Colour variation may be involved in species recognition, mating or camouflage (Parker, 2000; Shamim et al., 2014). One feature of lightly pigmented cuticles is translucency, a property that in some cases even allows internal structures to be seem through the exoskeleton. The Neotropical genus of bush-crickets

Phlugis (Stal, 1860), also known as “glass” or “crystal” bush-crickets (Chamorro-Rengifo and Braun, 2016), represent exceptional examples of cuticle translucency. Thus, species with a high level cuticle transparency represent alternative experimental systems in which cochlea-like organs can be accessed using non-invasive measurement techniques using light.

2.2. Materials and Methods

2.2.1. Fieldwork

This project was designed to study the properties of the hearing system across a diversity of tropical species, consequently investigating a broad auditory range. To collect specimens with such a broad range of sound frequencies, two expeditions to the Colombian rain forest were undertaken, visiting four different locations. Males and females of fifteen species were collected, these species represent a vast acoustic diversity ranging from moderately low (5-18 kHz) to ultrasonic (23-150 kHz) frequencies (Table 2.1). Additionally, specimens were also collected and selected based on the degree of cuticle transparency. Transparent cuticles was assume to be key feature for accessing the inner ear of the insect and measuring auditory activity in a non-invasive manner, using laser Doppler Vibrometry. Cuticle transparency was determined in the field by visual inspection of the forelegs, ranking them from high to low transparency (Table 2.1).

2.2.2. Sampling locations

Fieldwork was conducted between August 2014 and November 2015. Collecting sessions were done at night (18:00 – 24:00) along established trails

in the selected areas, with a total of 48 hours of sampling activity. Specimens were caught manually from understory vegetation and stored in 300 ml^3 plastic pots. Upon return to the base camp, the collected insects were placed in a wire mesh cylindrical cage and provided with food and water *ad libitum*. Three of the sampling locations were situated in the south-west region of Colombia and one in the northern part of the country (Figure 2.1).

Watershed Pericos: This Natural reserve is in the small community of El Salto, in the municipality of Buenaventura, Valle del Cauca (lat. 3° 56′N, long 76° 47′W). The watershed is located at the 76-km point of Route 40, the road that goes from the city of Cali to Buenaventura. Due to its location on the outskirts of the Andean western cordillera, this area is considered tropical rainforest and sub-Andean forest along an altitudinal gradient of 300–1,200 meters from the sea level. Annual precipitation falls between 4,000 and 10,000 mm. Temperature fluctuates daily between 18°C and 25°C (Cortes et al., 2010).

Costa Rica: This village is located in the municipality of Ginebra, Valle del Cauca (lat. 3°45'05.3"N, long. 76°13'57.8"W). The sampled sites are is situated on the western flank of the Andean central cordillera, and considered tropical lower montane wet forest with an altitudinal gradient between 1200–1500 meters from sea level. Rainfall average of approximately of 1,200 mm per year, and mean temperature fluctuates daily between 18°C and 24°C.

Bitaco reserve: The Bitaco reserve is located in the Chicoral community in the municipality of La Cumbre, Valle del Cauca (lat. 4° 35´ 56´´N, long. 77°

04´´51´´W). The reserve is situated on the medium flank of the Andean western cordillera; this area is considered tropical lower montane wet forest with an altitudinal gradient between 1700–2200 meters from sea level. Rainfall is approximately 2,000 mm per year, and daily temperature fluctuates between 14°C and 18°C.

Palmar de la Vizcaina: El palmar de la Vizcaina is an oil palm research centre surrounded by patches of tropical moist forest situated in the valley of the Magdalena river, 32 km from the municipality of Barrancabermeja, Santander (lat. 6°59'02.3"N; long 73°42'20.2"W). This locality has an elevation ranging between 85-95 m. Average annual temperature of 29.3°C, with an annual rainfall of 2693 mm, and relative humidity between 72-77%.

National Natural Park (PNN) Gorgona: PNN Gorgona encompasses the islands of Gorgona and Gorgonilla, and is situated at 35 km from the coast of Colombia (lat 2°47′ to 3°6′ N; long 78°6′, to 78°18′W). The park's ecosystem is classified as tropical wet forest with an area of 13.33 km^2, and a maximum elevation above sea level of 338 m. The average annual temperature is 26°C, and annual rainfall is 6891 mm.

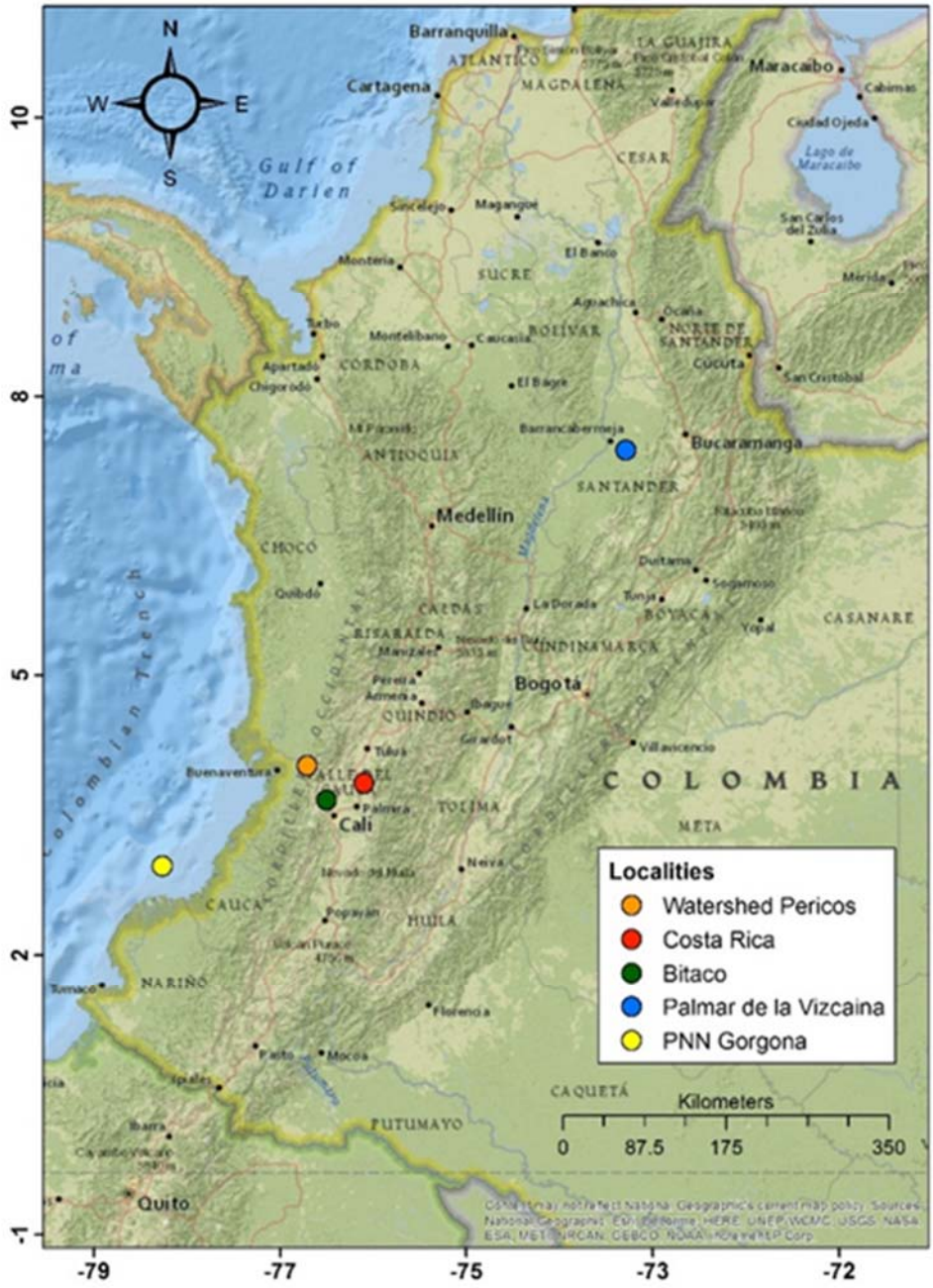

Figure 2.1. Map of Colombia and location of collection areas, which are represented by coloured dots.

2.2.3. Song recordings

The carrier frequencies of the calling song of some of the collected species were previously known, but in some cases (e.g. new species) it was necessary to perform recording sessions to establish the acoustic features of the male

calling songs before any auditory experiment was done. The recording took place in a sound-attenuated booth (internal length 1.8 m, width 1.8 m, and height 1.98 m), at room temperature (24–26°C) and relative humidity of 37–40%. The specimens were placed in a wire mesh cage at 10 cm from a 1/8" condenser microphone (G.R.A.S. 40DP), connected to a G.R.A.S. 26TC 1/4" Preamplifier (G.R.A.S. Sound & Vibration, Denmark). Data was stored on a laptop computer using an NI USB-6259 board (National Instruments, Austin, TX, USA) and NI-DAQmx components for LabVIEW SignalExpress software interface version 9.5.5 (National Instruments, Austin, TX, USA). Sound analysis was done using a custom-written software in Matlab version 8.4.0 (R2014b, Mathworks, Natrick, MA, USA). The carrier frequency was determined from the power spectrum of a fast Fourier transform calculated on a single call.

Table 2.1. Species collected during fieldwork, their calling song peak frequencies and cuticle transparency. The * indicates species which calling song was not previously known and recorded.

Species	Peak frequency (kHz)	Location	Cuticle transparency
*Copiphora vigorosa**	32.7	Cenipalma	Medium
Copiphora brevirostris	32	Pericos/Gorgona	Medium
Copiphora gorgonensis	23	Gorgona	Medium
*Subria nitida**	25	Cenipalma	High
Panacanthus pallicornis	5	Chicoral	Medium
*Nastonotus foreli**	23	Cenipalma	Low
*Gnathoclita sodalis**	18	Chicoral/Costa Rica	Medium
*Panacanthus lacrimans**	7	Pericos	Low
Neoconocephalus affinis	12	Costa Rica/Cenipalma	Low
*Phlugis poecila**	51	Cenipalma	High
*Supersonus pierci**	124	Pericos	Medium
Ca. Supersonus sp.*	56	Cenipalma	Medium
Acantharemus sp.*	22.7	Cenipalma	Medium
Artiotonus artius	42	Pericos	Medium
Docidorcercus sp.	22.5	Cenipalma	Low

2.2.4. Cuticle transparency measurements

Female and male adults of *Copiphora gorgonensis*, *C. vigorosa*, *Phlugis poecila*, *Neoconocephalus affinis*, *Nastonotus foreli*, and *Acantheremus* sp. (Figure 2.2) were taken from colonies reared at the University of Lincoln, UK.

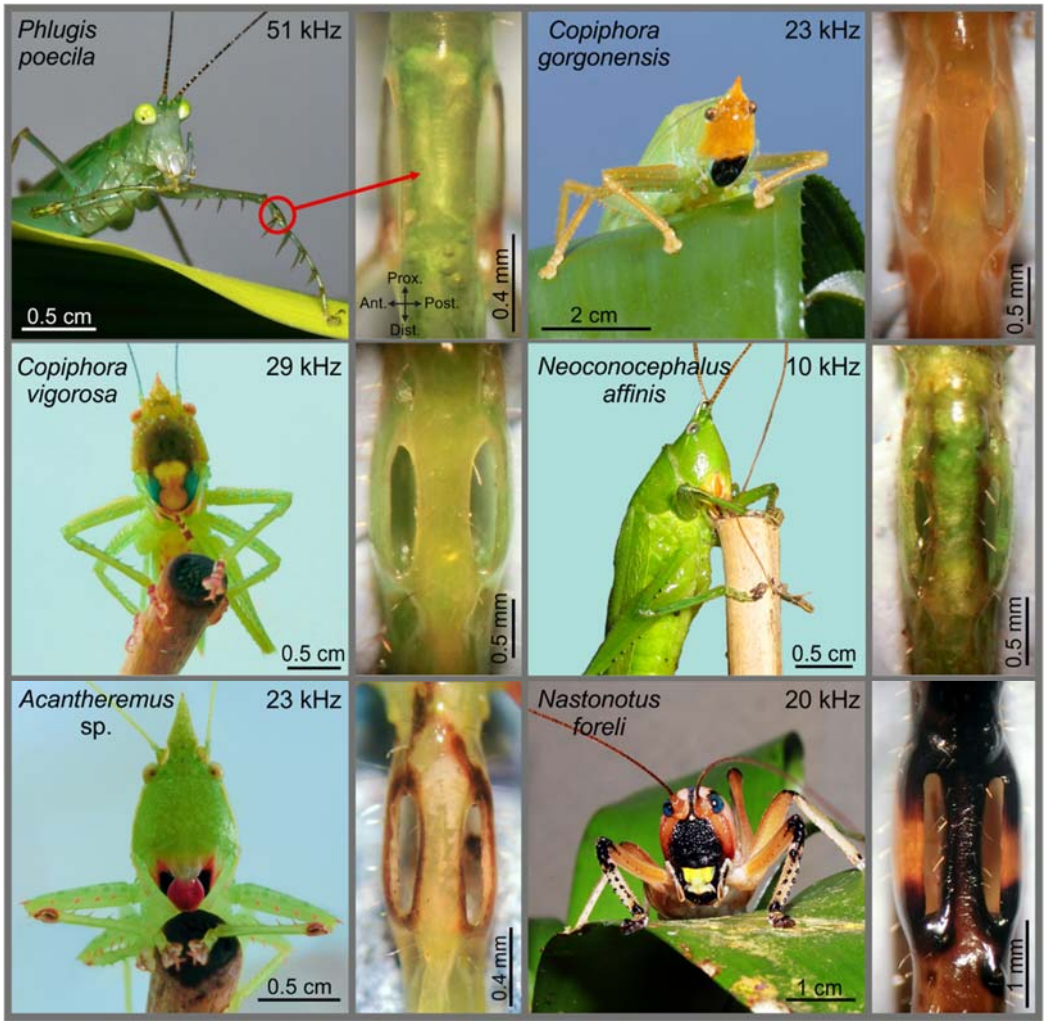

Figure 2.2. Species of bush-cricket (Tettigoniidae) used for the transmittance measurements. Right image: habitus of the species; Left image: close-up view of the ear region showing the colour and level of cuticle pigmentation for each species. Red circle indicates the position of the ear in *Phlugis poecila*.

Cuticle transparency was quantified by measuring the transmittance (ratio of the transmitted radiant flux to the incident radiant flux) of the cuticle covering the hearing organ. Cuticle samples were dissected from live specimens and

placed in a cavity well microscope slide containing insect saline solution (Fielden, 1960). A 50 μm diameter optic fibre connected to a spectrophotometer (USB2000 Fibre Optic Spectrometer, Ocean optics Inc., Oxford, UK) was placed on the projector lens in the camera ocular of a compound light microscope. For all the measurements a 40X objective lens was used and the reference light was the illumination system of the microscope (Halogen lamp), with brightness maintained at 5 volts consistently for all experiments (Figure 2.3). The spectrophotometer detector unit was connected to a computer via an USB port and the collected measurements were transformed into digital format using the OOIBase32 spectrophotometer operating software (Ocean optics Inc., Oxford, UK). The software calculates the percentage of energy passing through a sample relative to the amount that passes through the reference (equation 2.1).

$$\%T_{\lambda} = \frac{S_{\lambda} - D_{\lambda}}{R_{\lambda} - D_{\lambda}} \times 100\% \qquad (2.1)$$

Where $\%T_{\lambda}$ is the percentage of transmittance at wavelength λ, S_{λ} is the sample intensity, D_{λ} is the dark intensity, R_{λ} is the reference intensity (Ocean optics Inc., 2001).

For each transmittance measurement a reference spectrum was taken with the light source on and a blank (insect's saline solution in the cavity well of microscope slide) in the sampling region. The dark reference spectrum was taken with the light path blocked, and a stray light correction was applied using a boxcar pixel smoothing (spatial averaging applied to a spectrum) and time-

based averaging (10 averages). This processing removes noise by averaging the values of adjacent pixels and improves the signal to noise ratio at the expense of optical resolution (Ocean optics Inc., 2001).

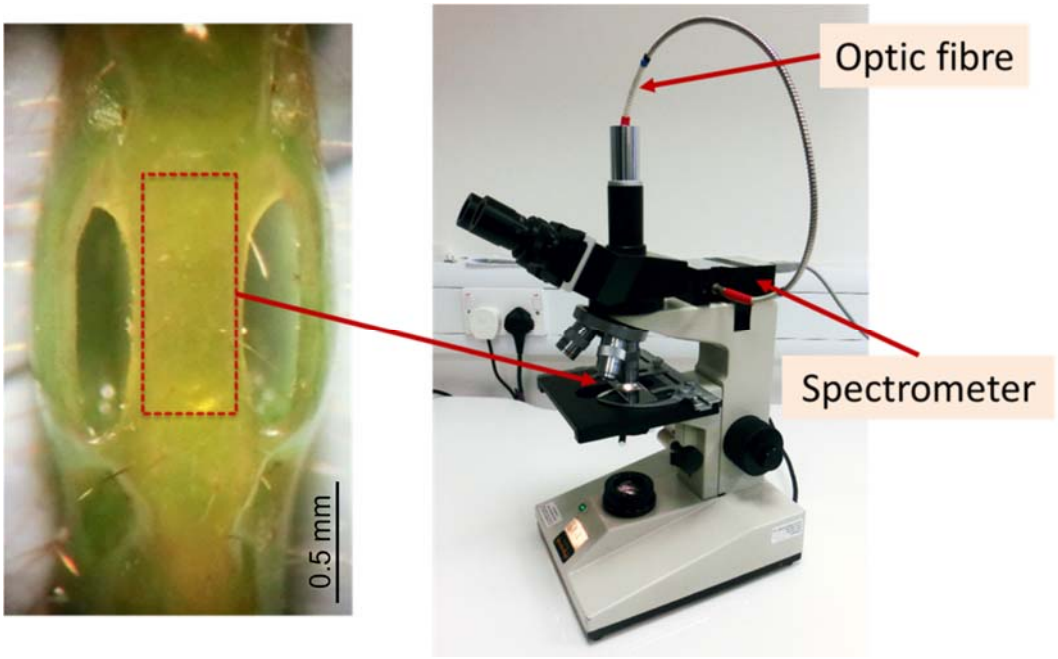

Figure 2.3. Cuticle transmittance measurement set-up. Cuticle samples were taken from the area demarcated by the red broken line rectangle and measured using the microscope-spectrometer arrangement indicated in the image.

2.2.5. Artificial actuator vibrations measured through transparent cuticle

A piece of freshly dissected cuticle from the dorsal ear area and a reference vibratory surface were used to evaluate the effects of the cuticle transparency on the laser Doppler vibrometry measurements, and to investigate whether the laser records ear vibrations on the cuticle, or on the *crista acustica* through the cuticle. Ear top cuticles were dissected from one of the forelegs of live specimens from all species, excluding *Ne. affinis,* and fixed with a mixture of beeswax (Fisher Scientific, Bishop Meadow Road, Loughborough, UK) and colophonium (Sigma-Aldrich, Dorset, UK) to the tip of a copper rod (0.632 cm

diameter and 23 cm long). Using a micromanipulator the external surface of sample was placed perpendicular between the laser head and the cone of a tweeter speaker enclosed in a custom made sound attenuation box (Figure 2.4). A 30 kHz pure tone was used as a reference signal and a 1/8" condenser microphone (Brüel & Kjaer, 4138-A-015 and preamplifier model 2670, Brüel & Kjaer, Nærum, Denmark) was positioned approximately 2-3 mm from the cuticle to monitor the acoustic isolation of the attenuation box and to ensure that the sound stimulus was not eliciting vibrations on the cuticle. The laser beam (633 nm, Polytec PSV-500; Waldbronn, Germany) was focused on the cuticle and a digital scanning grid of approximately 450 points was set on the dorsal surface of the piece of cuticle. The recording time for each of the measuring points was 32 ms (5 averages), with a sampling rate of 512 kHz. The vibratory response was measured in displacement after applying a 1 kHz high-pass filter. As a control, the cuticle was removed and the surface of the speaker was scanned using the same settings and grid of points. The effect of the cuticle on the laser signal was estimated by calculating the ratio between the displacement response of the laser beam through the cuticle and onto the vibrating surface, and the control displacement response (the beam directly onto the vibrating surface with no cuticle). In this way, higher laser displacement response ratios indicate that less laser signal is being blocked, or otherwise affected, by the beam travelling through the cuticle. Lower laser displacement response ratios indicate greater levels of the laser signal is being affected by the cuticle.

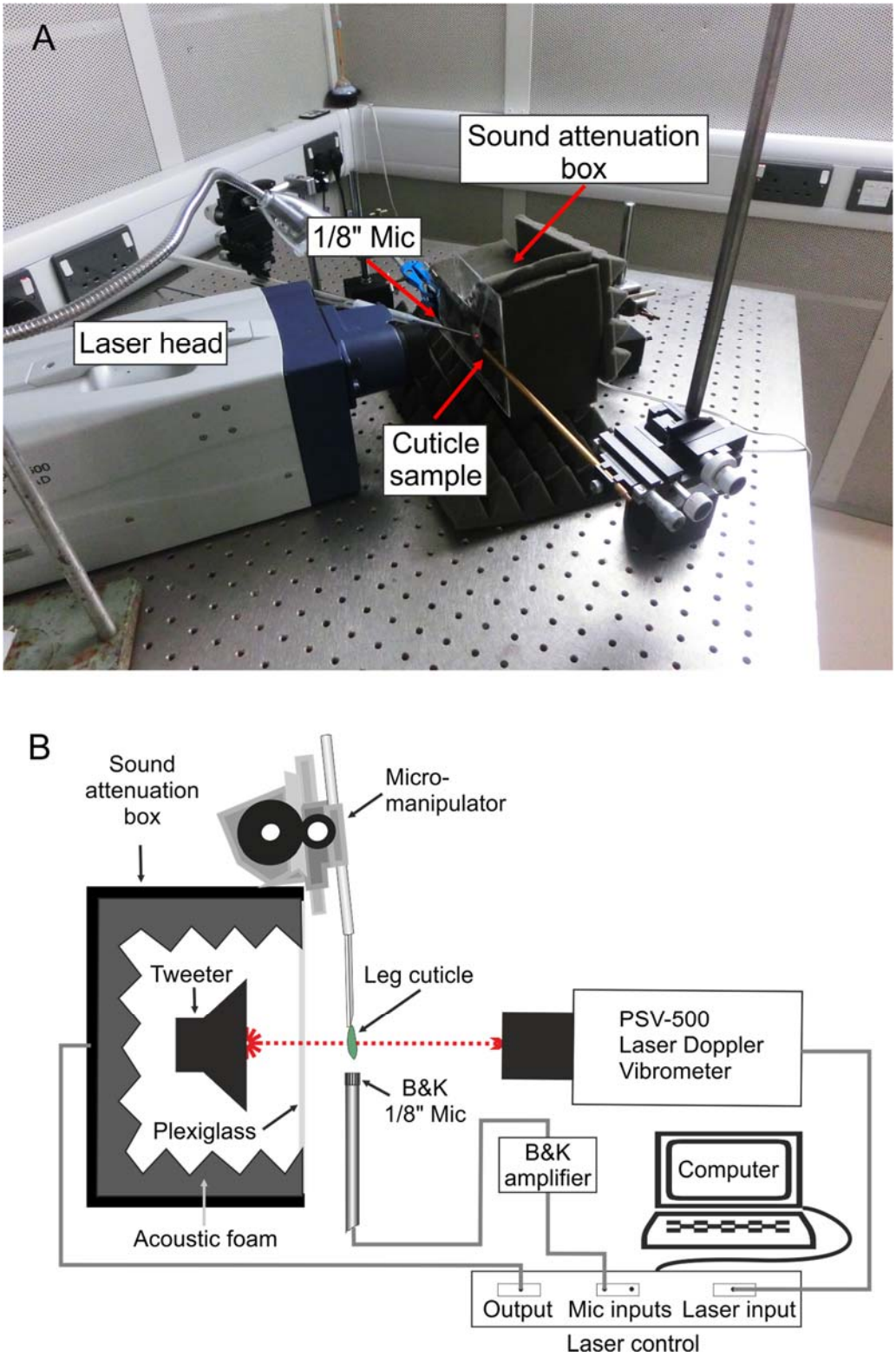

Figure 2.4. Experimental setup for obtaining laser response ratios from freshly dissected ear cuticle. A) Experimental setup for the measurement of vibrations through a piece of cuticle sample. B) Schematic diagram of the setup in image "A", image not to scale.

2.2.6. Cuticle thickness

Cuticle thickness was measured to evaluate the effects of this property on the laser signal response. For this, the previously dissected cuticle samples were cut transversally lengthwise down the midpoint of the sample. Samples were then placed on an aluminium scanning electron microscope (SEM) stub using carbon tape. Digital images were captured and analysed with an FEI Inspect S50 microscope (FEI, Hillsboro, OR, USA). Measurements were made with the graphics software Coreldraw X7 (Corel Corporation, Ottawa, Canada) using the dimension tool and adjusting the scale to real-world values using the scale bar from each individual SEM image (Figure 2.5).

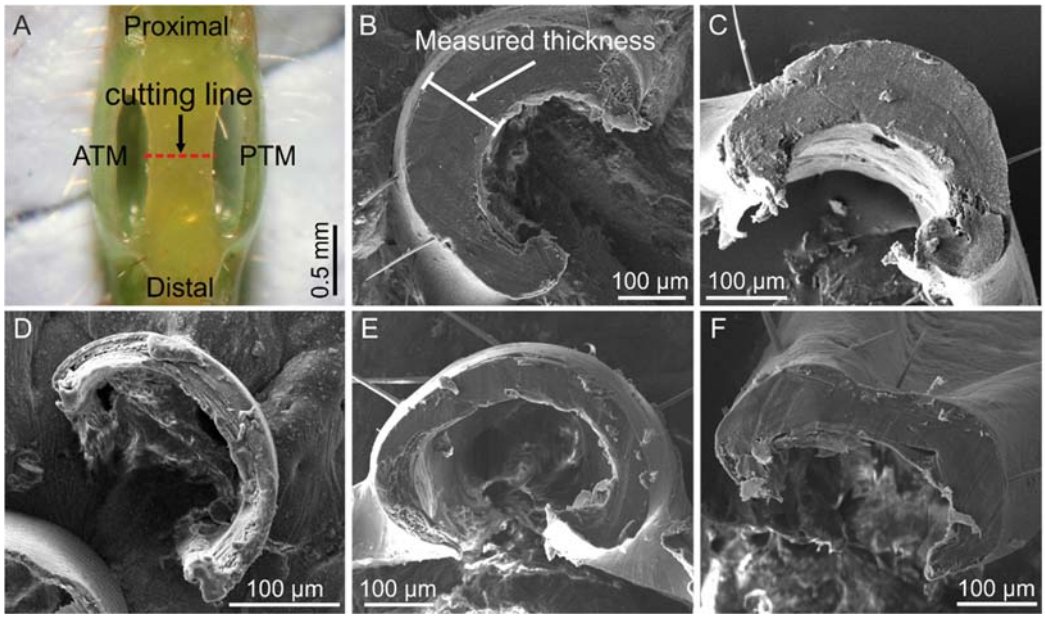

Figure 2.5. SEM images of cross section cuts of cuticle samples of the ear area. A) Dorsal view of the ear, red line indicates location of cross section dissection. B) *Copiphora vigarosa*. C) *Copiphora gorgonensis*. D) *Phlugis poecila*. E) *Acantheremus* sp. F) *Nastonotus foreli*. Modified from Sarria-S et al., 2017.

2.2.7. Data analysis

The relationship between laser response (a ratio), cuticular thickness (μm) and cuticular transmittance (%) was analysed using linear mixed effects (LMMs). Species was fitted as a random effect to account for species-differences in samples sizes. Parameters were logged before analysis. Models with and without interaction terms between cuticular thickness and cuticular transmittance were tested using likelihood ratio tests. The inclusion of the interaction significantly improved the model (χ_1^2 = 8.54, p<0.001).The relationship between cuticular thickness and transmittance was tested with a Pearson's correlation.

2.3. Results

2.3.1. Cuticle transmittance

Cuticle transparency was quantified across six species (Figure 2.2), and established the relationship between this property, cuticle thickness and LDV measurements of auditory activity. Using a spectrophotometer, cuticle transparency was quantified by measuring the transmittance (ratio of the transmitted radiant flux to the incident radiant flux) of the cuticle covering the hearing organ. Transmittance percentage values for all measured cuticles increased with wavelength in the visible light spectrum, 370-800 nm (Figure 2.6). At the light spectrum wavelength of the LDV laser (633 nm, Polytec PSV-500; Waldbronn, Germany) the values of the corresponding curves can be distinguished into two groups. One group with transmission values relatively high, *P. poecila*, and *C. gorgonensis* with averages of 73.73% ± 3.10 and 59.93% ± 4.15 respectively (mean ± s.e., Figure 2.7). The second group

includes values below 50% and it is formed by *C. vigorosa*, *Acantheremus* sp., *N. affinis*, and *N. foreli* with transmission percentages of 40.00% ± 3.24, 34.14 ± 12.24, 33.46% ± 2.32, and 18.82% ± 2.64 respectively (mean ± s.e.).

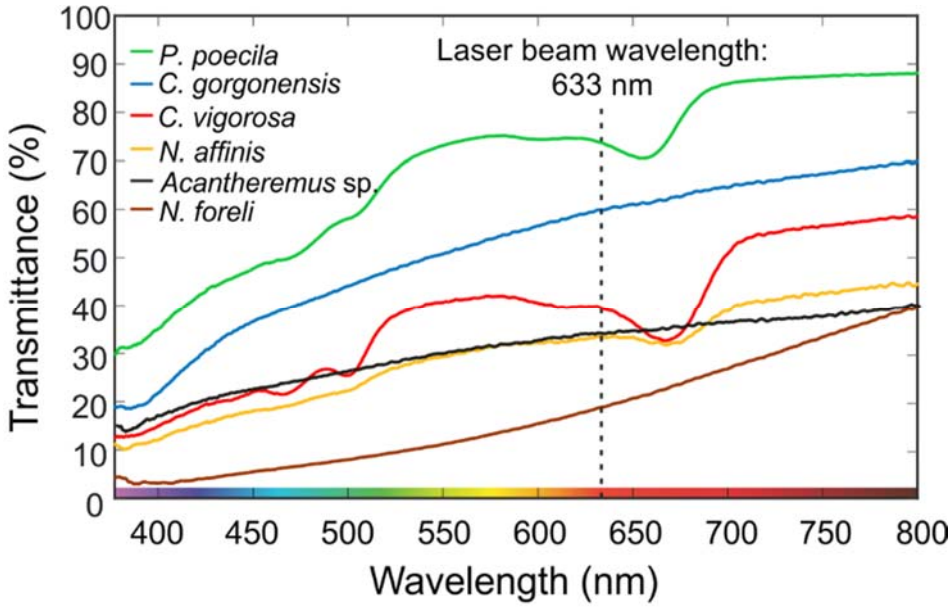

Figure 2.6. Cuticle transmittance values for all species studied. Transmittance curves percentage of light diffused through the ear dorsal cuticle measured in the visible light spectrum (370–800 nm). Modified from Sarria-S et al., 2017.

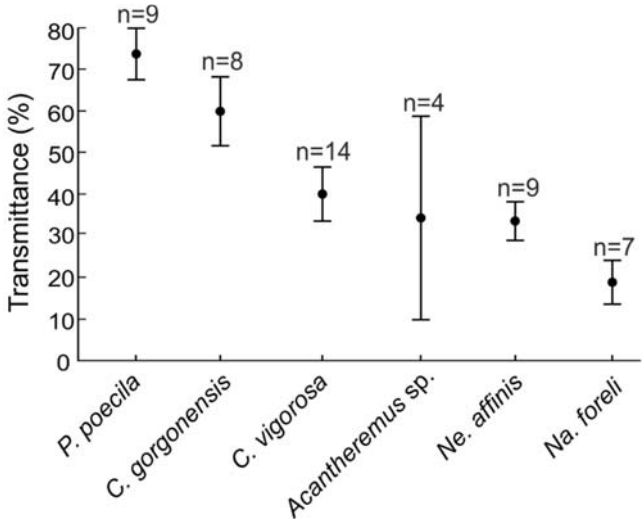

Figure 2.7.Mean transmittance values (± s.e.) of the ear dorsal cuticle of all species at the laser beam wavelength (633 nm). Modified from Sarria-S et al., 2017.

2.3.2. Laser Doppler vibrometry ratio response

The effect of cuticle transparency specifically in relation to transmission of light from a LDV was calculated as a ratio of the LDV response (measured as displacement) from a reference vibrating surface (a membrane on a speaker playing a sine wave, Figure 2.4), and the same surface as measured through a sample of ear cuticle (Figure 2.8). The relationship between the LDV response and cuticle transmission, including cuticle thickness, was quantified through linear regression of these variables. Cuticle thickness was obtained by measuring cross sections of dissected ear cuticle (Figure 2.5). A linear mixed effect model found that laser displacement response (L_r) was significantly related to the interaction between cuticle thickness and transmittance values (LMM: cuticular thickness x transmittance β±SE=0.90±0.31, $F_{1,18.07}$=8.53, P=0.009; LMM: cuticular thickness β±SE=-3.52±1.11, $F_{1,16.13}$=9.96, P=0.006; LMM: transmittance β±s.e.=4.08±1.30, $F_{1,18.07}$=9.82, P=0.006). Lowest laser displacement response (L_r) occurred when both the cuticle was thick and when transmittance was low (Figure 2.8); the strongest laser displacement response (L_r) occurred when transmittance was high and cuticles were thin (*P. poecila:* mean±s.e. =-0.24±0.07). Transmittance and cuticle thickness were not correlated (r_p=-0.09, P=0.667).

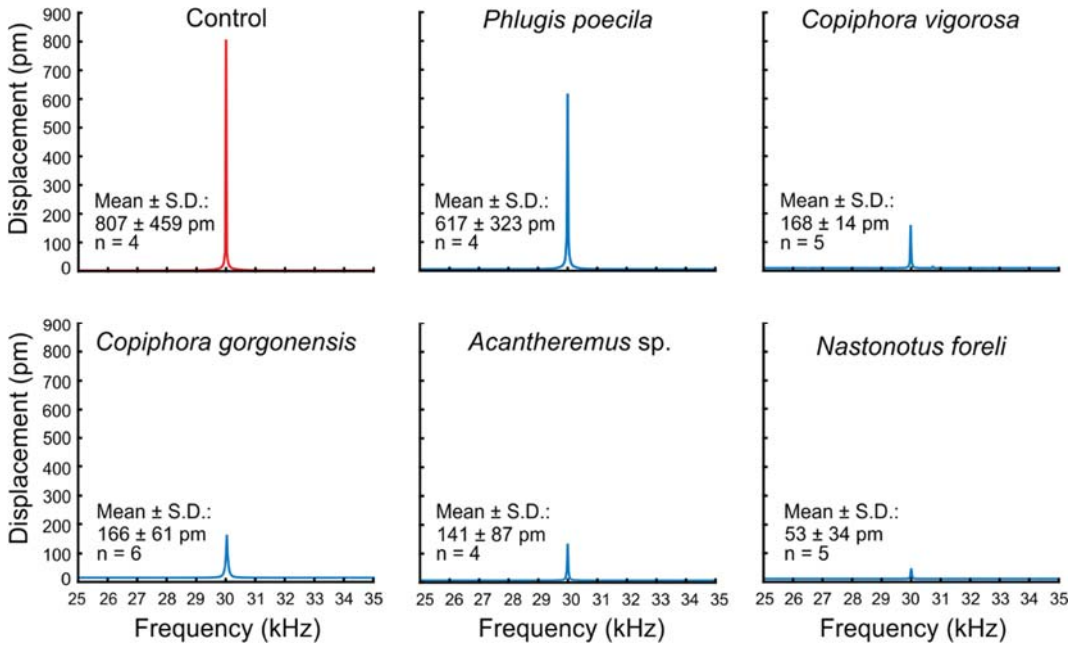

Figure 2.8. Laser measurements of the vibrating surface through a piece of cuticle. In red colour is the average laser signal response of the control measurements. In blue the average laser signal response through a cuticle sample from five species.

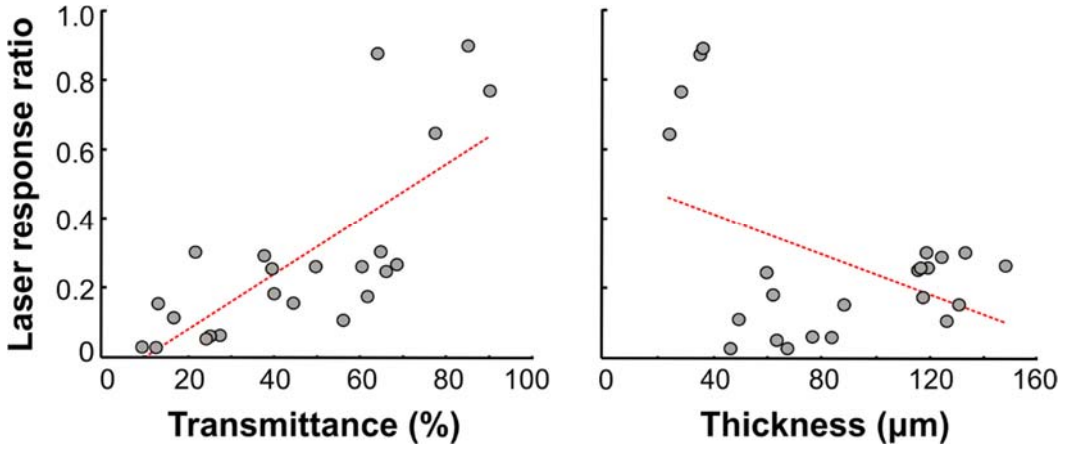

Figure 2.9. Relationship of cuticle transmittance, cuticle thickness and laser response ratio.

2.4. Discussion

Light reflectance and transmission through the insect cuticle has been measured previously with the purpose of elucidating a vast number of aspects related to vision, light emission, iridescence of wings or other body parts (Giraldo et al., 2016; Kim et al., 2012; Parker and Martini, 2006; Seago et al.,

2009). Here, it is reported for the first time, the association of cuticle transparency with audition in an invertebrate. The results indicated that species that present cuticles with transmittance values above 60% are suitable for LDV measurements and this gives support to Montealegre-Z and Robert assumptions about cuticle transparency which were based on non-systematic observations (Montealegre-Z and Robert, 2015).

From the studied species, *P. poecila* can be described as a good alternative for auditory research due to its cuticle transparency and hearing capabilities. *P. poecila* is characterised by presenting a light green coloured cuticle with high transmittance value. This feature could have been an adaptation for this species' predatory behaviour and environmental context. Unlike other bush-crickets, this species exhibits diurnal activity and it is found dwelling on leaves of bushes where it hunts for small arthropods. Being active during the day means it has to be highly adapted to compete in an environment where predators and preys have highly developed sight. Consequently, in order to be able to hunt and at the same time be inconspicuous, the adoption of a translucent green coloration reduces the chances of contrasting with the background under the sun light, a similar strategy used by the green beetle *Calloodes grayanus* (Mckenzie and Large, 1998). Although, *P. poecila* has diurnal activity which suggests good vision, this species also has a well-developed hearing system. Like other bush-crickets, *P. poecila* males produce calling songs to attract females. The calls are broad band acoustic signals between 30 and 90 kHz with a main carrier frequency peak around 50 kHz (Appendix A1, Figure A1.2A-C). Therefore, it is likely that hearing capabilities

in this species incorporates a wide spectrum of frequencies from the audible to the ultrasonic scale.

It was also confirmed that cuticle transparency and cuticular thickness are primary factors allowing the non-invasive measurement of auditory activity in the bush-cricket inner ear. Furthermore, the analysis reveals that transmittance of light through the cuticle is a reliable indicator of a species suitability for experiments specifically using LDV. The lack of correlation between cuticle transmittance and thickness indicates that pigmentation affects transparency, and in turn, laser measurements. This explains why established model species in insect hearing research like *Mecopoda elongata* (Palghat Udayashankar et al., 2012) were not suitable for non-invasive laser measurements.

Chapter 3: Travelling waves

Parts of this chapter have been published in:

Sarria-S, F. A., Chivers, B. D., Soulsbury, C. D., & Montealegre-Z, F. (2017). Non-invasive biophysical measurement of travelling waves in the insect inner ear. Royal Society Open Science, 4(5), 170171.

Contributions: My participation on this paper consisted in the realization of field work for specimen's collection, designing and running laboratory experiments, data collection and analysis, and writing and editing the manuscript.

In order to corroborate the effect of cuticle transparency on laser Doppler vibrometry measurements *in vivo*, and to present novel data on travelling wave from a purely non-invasive methodology, the auditory activity of *P. poecila* specimens were measured, considering from chapter 2 that this species presented the highest transmittance values.

3.1. Introduction

Among vertebrates, mammals and birds exhibit an elaborate hearing system, in which auditory perception relies on mechanical and neurophysiological processes occurring in the fluid-filled cochlea (Dallos, 1992). Sound waves transferred during the impedance conversion stage generate pressure differences between the cochlear chambers. These compression changes cause vertical movements, which travel along the basilar membrane (BM) carrying mechanical energy instead of acoustic energy (Nobili et al., 1998). These oscillations, known as travelling waves (TWs), are a key element for frequency analysis and signal transduction (Robles and Ruggero, 2001). TWs spread along the BM with a wave amplitude gradually increasing towards a resonant location (Patuzzi, 1996; Russell and Nilsen, 1997b). The maximum

displacement stimulates the hair cells located on the organ of Corti, through the mechanical opening and closing of ion channels (Ashmore, 1992; Dallos et al., 1982; Roberts et al., 1988; Vater and Kossl, 2011).

It has been observed in cochleae of guinea pigs, chinchillas, squirrel monkeys, and cats (Robles and Ruggero, 2001; Robles et al., 1986) in which the BM in these mammals high frequencies produce resonances at basal regions and low frequencies near to the apex, with intermediate frequencies being analysed between these two extremes (Russell and Nilsen, 1997b; Young, 2007). This spatial frequency distribution, also known as tonotopy, was previously attributed to the BM stiffness gradient alone (Dallos, 1992), but it has been argued that the place associated frequency decomposition is the product of the interaction between the BM's anisotropic properties and the active processes of the hair cells, Reissner, and tectorial membranes (Naidu and Mountain, 1998). Although the active and passive processes are widely accepted, it has being difficult to demonstrate their integration experimentally (Young, 2007). Additionally, the spatial frequency response of the BM has been calculated indirectly through computational models or estimated from auditory afferent nerve fibres at selected points along the BM (Elliott and Shera, 2012; Russell and Nilsen, 1997b).

First used to describe the motion of the basilar membrane in the cochleae of human cadavers (von Békésy, 1960), passive travelling waves are viewed today as the substratum for active cochlear amplification in mammals (Mammano and Nobili, 1993; Robles and Ruggero, 2001). Phenomena

analogous to travelling wave have been directly observed in the basilar papilla of birds (Gummer et al., 1987) and the ears of bush-crickets (Montealegre-Z et al., 2012; Palghat Udayashankar et al., 2012), and have also been inferred, via the timing of responses of auditory-nerve fibres, in the hearing organs of some reptiles and frogs (Hillery and Narins, 1984; Smolders and Klinke, 1986).

Existing experimental approaches to infer TW function in vertebrate and bush-crickets are inherently invasive, compromising the fine-scale mechanics of each system (Hummel et al., 2016; Palghat Udayashankar et al., 2012). As it was demonstrated in the previous chapter, bush-crickets with transparent ear cuticles (Figure 3.1) are potential model species for direct, non-invasive measuring of auditory activity.

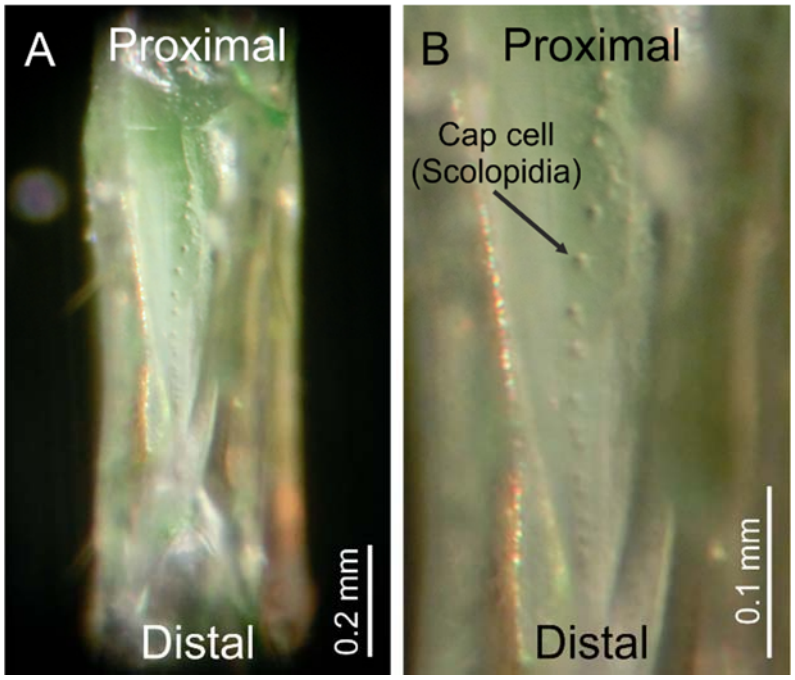

Figure 3.1. Cuticle transparency in a glass bush-cricket *Phlugis* sp. A) Dorsal view of the tibia, the tympanal organ is clearly visible through the cuticle without further manipulation of the specimen. B) Close-up view of the hearing organ. The cap cells (scolopidia) are visible through the cuticle.

3.2. Methods

3.2.1. Mounting the specimens for LDV measurements of travelling waves

Protocols for measuring ear activity with LDV follows Montealegre-Z *et al*. (Montealegre-Z et al., 2012). For the LDV experiments, insects were initially anesthetized with a triethylamine-based mix (FlyNap®, Carolina Biological Supply Company, Burlington, North Carolina, USA) to facilitate the fastening to a horizontal brass platform (5 mm wide, 1 mm thick and 70 mm long). The dorsal pronotal area and legs, except for the frontal pair, were fixed to the platform using a mixture of beeswax (Fisher Scientific, Bishop Meadow Road, Loughborough, UK) and colophonium (Sigma-Aldrich, Dorset, UK). The front legs were restrained using brass wires, which allowed positioning of the tibia and femur in a 90 degrees angle. Additionally, the brass plate was attached to an articulated aluminium rod (150 mm long, 8 mm diameter) allowing the dorsal surface of the ear to be placed perpendicular to the scanner's laser beam. All experiments were carried out inside a sound-attenuated booth (internal length 2.40 m, width 1.8 m, and height 1.98 m), at room temperature (24–26°C) and relative humidity of 32-35%. The scanning head of the laser and the experimental setup were placed on a Melles Griot Optical Table Breadboard, Pneumatic Vibration Isolation (1m x 1m area) (Melles Griot, Rochester, NY).

3.2.2. Laser Doppler Vibrometry measurements of travelling waves

The sound-induced vibration pattern of the inner ear was measured using a micro-scanning laser Doppler vibrometer (Polytec PSV-500; Waldbronn,

Germany) fitted with a close up attachment. The mounted specimens were positioned so that the cuticle overlaying the ear was perpendicular to the lens of the laser unit. A loudspeaker was positioned 30 cm, ipsilateral to the specimen to broadcast the sound stimulus (Figure 3.2). Periodic chirps were used as the acoustic stimulus and were generated by the Polytec software (PSV 9.0.2), and passed to an amplifier (A-400, Pioneer, Kawasaki, Japan), and sent to a loudspeaker (Ultrasonic Dynamic Speaker Vifa, Avisoft Bioacoustics, Glienicke, Germany). The periodic chirps contained frequencies between 5 and 80 kHz, and the stimulus was flattened so all frequencies were represented at 60 dB ±1.5 dB (SPL re 20 μPa) at the position of the ear. A B&K 1/8 inch microphone was placed at the position of the ear to monitor and record the acoustic stimulus at the position of the ear as a reference (Figure 3.2). The laser system was used in scan mode. A grid of scan points on the dorsal surface of the CA was established using the PSV 9.2 acquisition software (Polytec, Waldbronn, Germany). Depending on the size of the insect's leg, the actual number of measuring points per grid varied among specimens, with ~800 points per ear. Within the frequency domain setting of the vibrometer, a frequency spectrum was calculated for each point using a FFT with a rectangular window, at a sampling rate of 256 kHz samples/second, 64 ms sampling time with a frequency resolution of 15.62 Hz. A high-pass filter of 1 kHz was applied to the both the vibrometer and reference microphone signals during the scanning process.

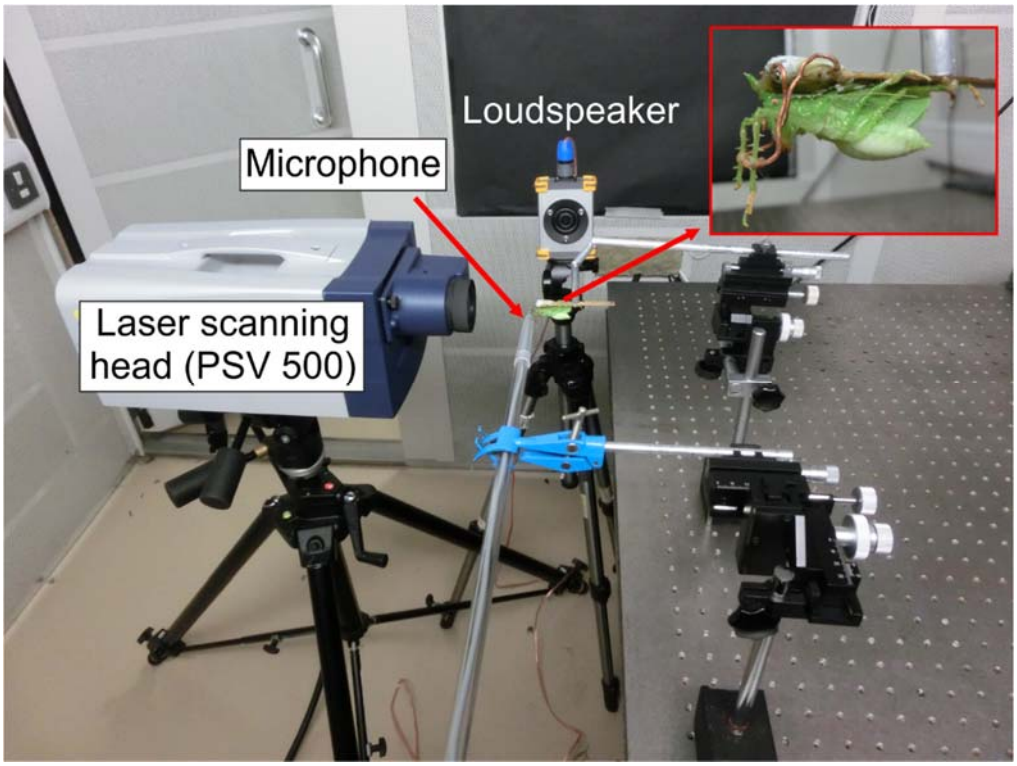

Figure 3.2. Experimental set-up for non-invasively measuring travelling waves in bush-crickets. Inset: preparation of the mounted bush-cricket.

3.2.3. Data analysis

Data from all scanned points were examined using the PSV 9.2 presentation software (Polytec, Waldbronn, Germany). Frequency spectra, ear displacement animations, and oscillation profiles were produced for selected frequencies within the recorded range. Frequency spectra of the vibrometry data were normalised to those of the reference signal by computing the transfer function of the two (Windmill et al., 2005). For the TW analysis, coordinates and displacement values from points corresponding to a 1 mm profile line set distal to proximal on the measured grid (Figure 3.3) were exported as an ASCII file. The obtained data points were analysed using a custom Matlab code (Matworks Inc., Nauticks, USA), which generates plots of the TW recorded from the scanned ears. The plots allowed us to visualise and

measure the velocity response of each point in the frequency domain. The graphical representation was used to evaluate two of the TWs' criteria: asymmetric envelope and phase lag (Windmill et al., 2005). Furthermore, TWs' propagation velocity and wavelength were calculated from the phase response using equations 3.1-3.3.

$$\delta_t = \frac{\delta_\phi}{2\pi f} \tag{3.1}$$

$$V_{wave} = \frac{\delta_x}{\delta_t} \tag{3.2}$$

$$\lambda = \frac{2\pi\delta_x}{\delta_\phi} \tag{3.3}$$

Where *f* is wave frequency (Hz), δ_ϕ is phase difference (rad) between two points at different locations, δ_t is the travel time (s), δ_x is the distance travelled (m), V_{wave} is wave velocity and λ is wavelength (Robles and Ruggero, 2001; Windmill et al., 2005). The relationship between these parameters and frequency was tested using LMMs. In each model, individual identification was fitted as a random effect. For all LMMs, degrees of freedom were calculated using Sattherwaite's approximation. Statistical analysis was carried out using the lme4 package (Bates et al., 2014) run in R version 3.3.1 (R Development Core Team, 2016).

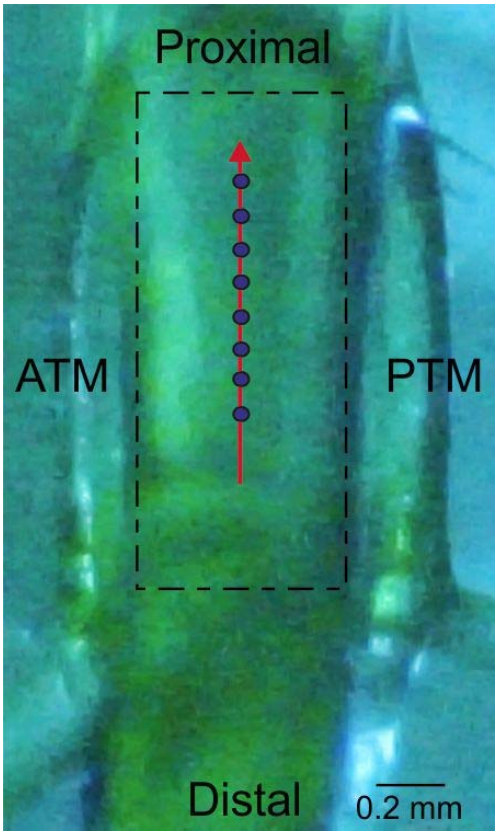

Figure 3.3. Scanning point grid on the dorsal cuticle of the ear. Profile line (in red) set in a distal to proximal direction. Coordinates and displacement values from points on the line were exported as an ASCII files for further calculations.

3.3. Results

3.3.1. *In vivo* measurement of travelling waves

In order to corroborate the feasibility of transparent species for *in vivo* audition experiments, the auditory activity of specimens of *P. poecila* were investigated as this species presented the highest transmittance values and thinnest cuticles. Non-invasive measurements of tonotopy and TWs *in vivo* were done by directly measuring the sound-induced vibration pattern of the ear using LDV (Figure 3.4 and 3.5).

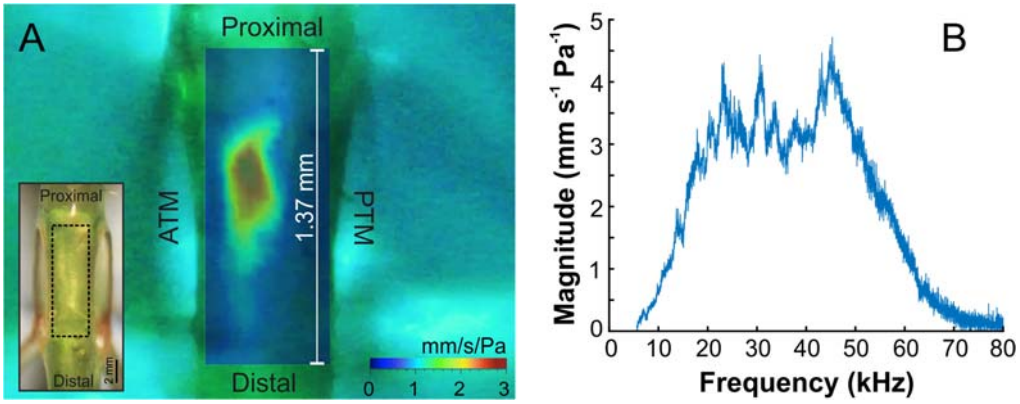

Figure 3.4. Auditory response of *Phlugis poecila* inner ear. A) Laser vibration map showing the distribution of areas of high vibration amplitude. Inset: ear area scanned during the LDV experiments. B) Auditory response of the crista acustica to acoustic stimuli.

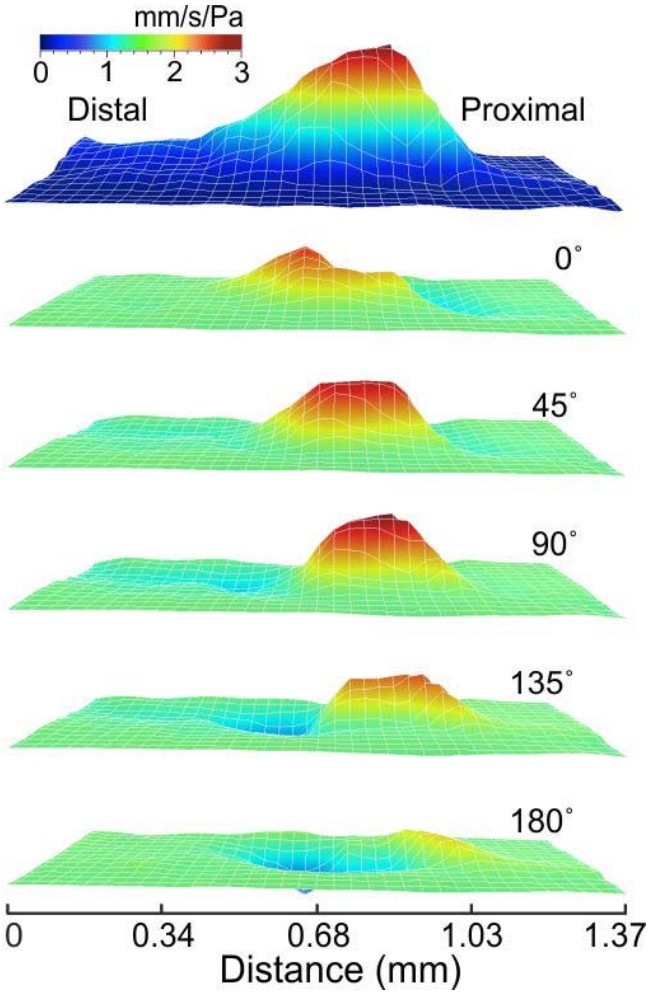

Figure 3.5. 3D representation of travelling wave. Same data as in Figure 3.4, ear response to a 10 kHz through phases of 45 degrees of the oscillation cycle.

3.3.2. Tonotopy

A spatially discrete response for frequencies between ~10 and ~60 kHz was observed from non-invasive measurements along the length of the hearing organ (Figure 3.6 A-D). With increasing stimulus frequency, the maximum response shifts towards the distal part of the leg (Figure 3.6 B-D). This tonotopic response was only observed in the tympanal organ, as oppose to measurements of the tympanal membrane demonstrated that this structure has a type 1 mode oscillation, typical of a two-dimensional elastic membrane under tension (Fletcher, 1992), and with a wide bandwidth (2-60 kHz) response to the acoustic stimuli (Figure 3.7).

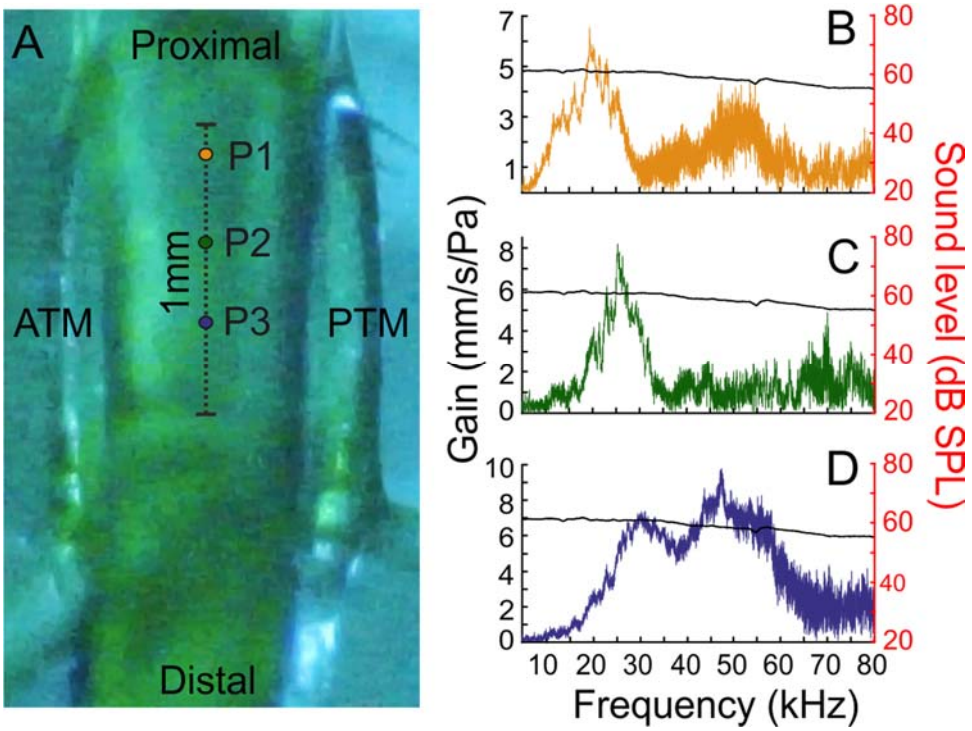

Figure 3.6. Spatial frequency mapping and travelling waves in the inner ear of *Phlugis poecila*. A) Close up view of the left leg ear showing a three-point transect on the ear area between the anterior (ATM) and posterior tympanal membrane (PTM). The locations where the maximum displacement were recorded in the ear for 19 kHz, 25 kHz, and 47 kHz are represented by P1, P2, and P3 respectively. B-D) Frequency response measured as velocity gain at locations P1-P3. Black line represents the amplitude spectrum of the stimulus signal.

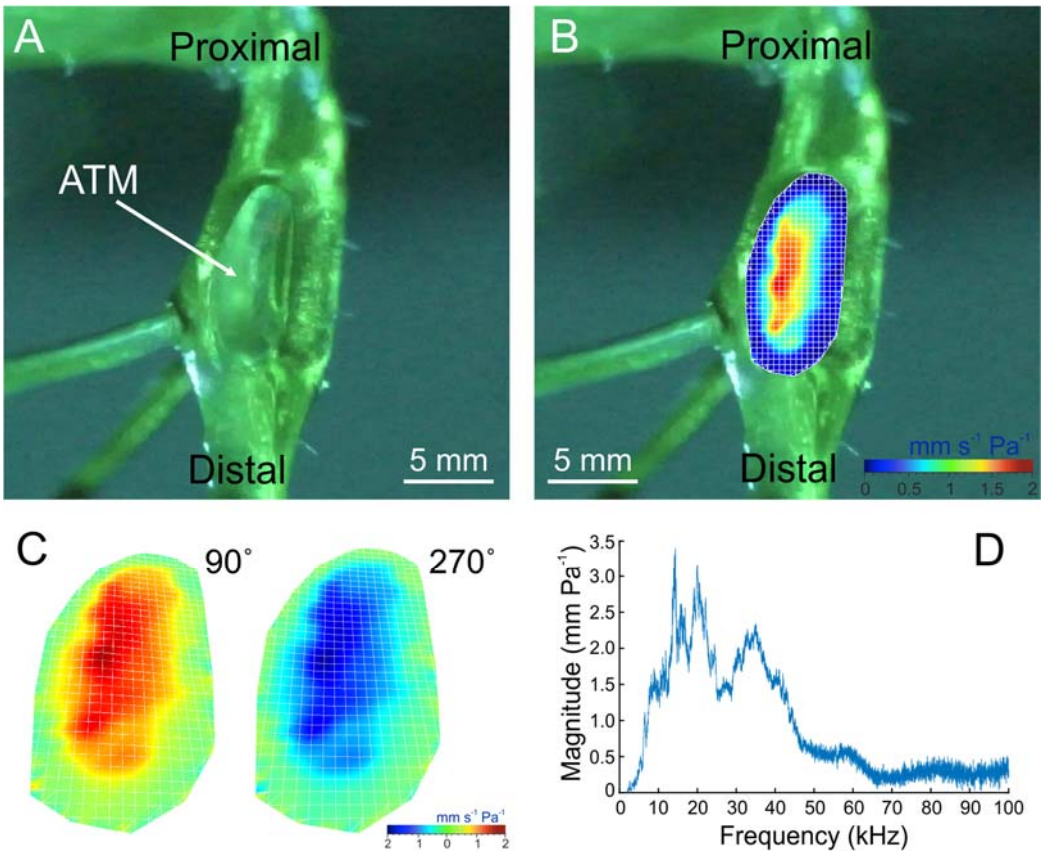

Figure 3.7. Tympanal membrane compliant response in *Phlugis poecila*. A) Anterior tympanal membrane (ATM). B) Scan points and magnitude response of the tympanal membrane to acoustic stimuli. C) Tympanal membrane amplitude deflections at 90° and 270°. D) Spectrum response of the tympanal membrane to a bandwidth from 2 kHz to 100 kHz.

The measured response in the inner ear satisfies two criteria for the inference of TWs: (i) asymmetric envelope and (ii) phase lag (Robles and Ruggero, 2001). The magnitude of *crista acustica* displacement shows an asymmetric envelope around the point of the maximal deflection. This point is also the location where the wave is seen to compress before dying off.

TW asymmetry was evaluated as the response gain (mm s^{-1} Pa^{-1}) along a transect line across the *crista acustica* for different frequencies (Figure 3.8 A-C) and it was observed that the position of the maximum displacement of the

TW envelope varies with frequency. At 19 kHz the wave is asymmetrical at a distance of 720 µm (Figure 3.8A), at 25 kHz the asymmetry occurs around 577 µm (Figure 3.8B), and for 47 kHz the same phenomenon is observed approximately at 447 µm (Figure 3.8C). Similarly, the phase response across the *crista acustica* displays an increasing lag along the transect (Figure 3.8A-C). The lag increases as a function of frequency; for instance, at 19 kHz the phase lag is 281°, while at 47 kHz the lag reaches 419° difference between the initial and final phase angle.

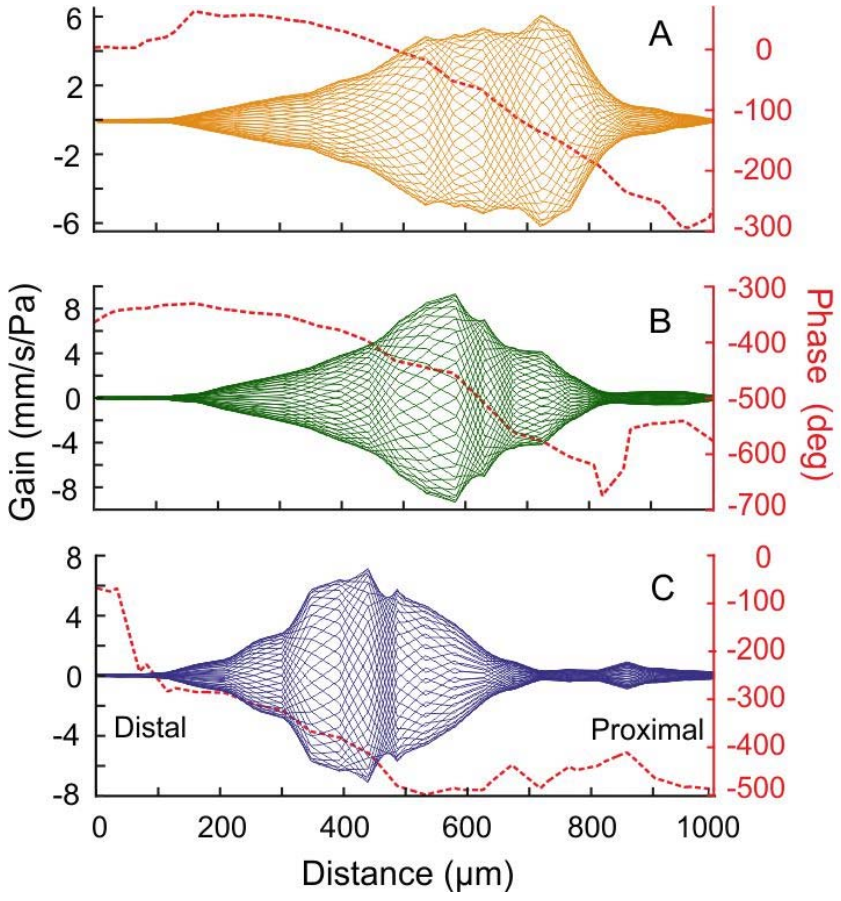

Figure 3.8. Envelope and phase reconstruction along the transect in figure 3.6A for A) 19 kHz, B) 25 kHz, and C) 47 kHz. The deflection envelopes are constructed by displaying phase increments of 10° in the full oscillation cycle. The red colour broken line represents the phase lag in degrees (red scale in the right) for the same frequencies and distance.

3.3.3. Travelling wave velocity and wavelength

Velocity and wavelength of propagation are parameters of TWs that can be accurately characterised with our approach. The velocity of the TW in the inner ear of *P. poecila* increased from 6.22±1.22 m s^{-1} to 18.55±3.04 m s^{-1} in a frequency range of 10 kHz to 50 kHz. The wavelength on the other hand decreased from 0.62±0.12 mm to 0.37±0.06 mm for the same frequency range. In our measurements, TW's velocity was significantly positively related to sound frequency (LMM: β±SE=0.31±0.02, $F_{1,103}$=315.60, P<0.001; Figure 3.A). Conversely, there was a significant decrease in wavelength as frequency increased (LMM: β±SE=-0.006±0.001, $F_{1,103}$=77.48, P<0.001; Figure 3.9B).

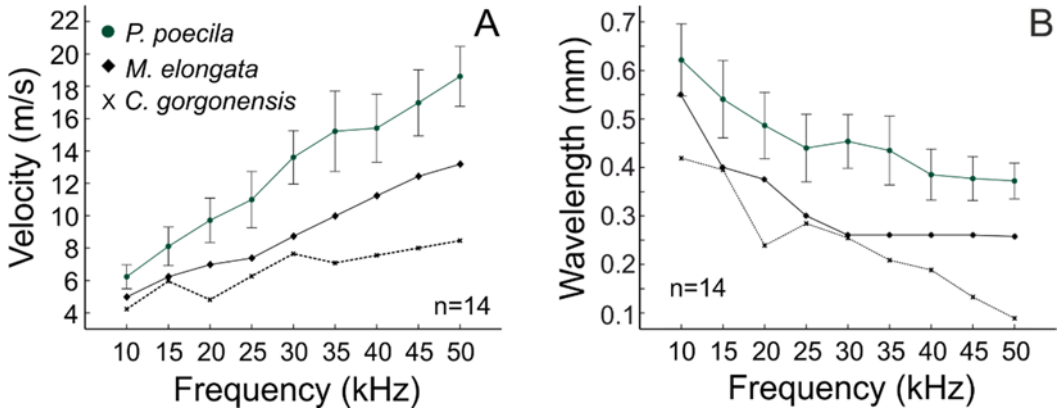

Figure 3.9. Correlation between frequency and travelling wave's velocity and wavelength. A) The velocity of the travelling wave in *Phlugis poecila, Mecopoda elongata, and Copophora gorgonensis*. B) Travelling-wave wavelength in *Phlugis poecila*, *Mecopoda elongata*, and *Copophora gorgonensis*.

3.4. Discussion

Taking advantage of the high level of cuticle transparency (Sarria-S et al., 2017) and wide frequency bandwidth of auditory perception in *Phlugis poecila* (Figure 3.4B and Figure 3.7D), it was corroborated the use of bush-crickets as an alternative system for the study of auditory processes. The observed phase

lag and asymmetric envelope along the *crista acustica* (Figure 3.8A-C) allowed to characterise the auditory response as a travelling wave with displacement maxima at tonotopically specific locations. The obtained travelling wave velocities and wavelengths are shown (Figure 3.9A-B). These parameters have been calculated in the bush-cricket *Mecopoda elongata* by opening the cuticle and draining the original auditory vesicle fluid. The data presented here was collected non-invasively from an intact system, reducing the effects of surgically opening the inner ear cavity e.g. changes in the hydrostatic pressures and fluid density (Montealegre-Z et al., 2012; Montealegre-Z and Robert, 2015).

It has been shown that the amplitude velocity of the crista acustica decreases rapidly when the system is altered by, for example, vacating its fluid, that this operation causes also alters the phase of the associated tympana (Montealegre-Z and Robert, 2015). However, the decrease in TW wavelength with increasing frequency, and the corresponding increase in TW velocity, presented here is in good agreement with predictions of TW function as observed in vertebrate (Robles and Ruggero, 2001; Şerbetçioğlu and Parker, 1999) and invertebrate (Montealegre-Z et al., 2012; Palghat Udayashankar et al., 2012) models.

Understanding hearing processes such as tonotopy and TWs in mammals is crucial to further auditory research regarding nonlinear processes within cochlea (Elliott and Shera, 2012). As mentioned before, anatomical limitations for accessing and obtaining data *in vivo* and in an intact system has been a

major drawback in this field. Recently, methods for the measurement of auditory activity *in vivo* have improved notably for mammals. Developments with a technique using optical coherence tomography (OCT) provides a visual method for depth-resolved displacement measurements of TWs through the bony shell that protects the cochlea (Lee et al., 2015; Warren et al., 2016). Although such OCT techniques appear to be non-invasive, it still requires the middle ear bulla to be surgically treated to allow visual access to the cochlea. On the other hand, attempts to relate the biomechanical tonotopy to the frequency tuning of the corresponding sensory cells in bush-crickets have produced important advances in this field. It was showed recently that a significant difference between mechanical tuning of the crista acustica motion and neuronal tuning of sensory cells (Hummel et al., 2016). These measurements, revealed a biomechanical filter process that considerably sharpens the neuronal response. With techniques available at the moment, these experimental procedures are still invasive, requiring opening the tympanal organ and draining the AV fluid. Bush-cricket ears with transparent cuticles are an exciting alternative approach to learn more on these micro-scale ears and on complex auditory process impossible to measure in other system (Montealegre-Z et al., 2012; Palghat Udayashankar et al., 2012).

Chapter 4: The chemical composition of the inner ear liquid

The purpose of this chapter is to examine the ear fluid of different bush-cricket species with the aim of demonstrating, from a biochemical perspective, that the liquid contained in the auditory organ has a specific ionic composition necessary for enhancing the hearing process. This study serves as a preliminary assessment both from the point of view of number of species investigated and the number of individuals per species. However, the obtained results herein offer the bases to further research in bush-crickets with comparable hearing mechanism and biochemical traits to those found in vertebrates.

4.1. Introduction

Acoustic perception in animals involves the mechanical transformation of sound into neural signals (Torres and Giráldez, 1998). This mechanoelectrical transduction process takes place in the inner ear, where the auditory receptor cells are immersed in a specialized liquid environment that enables and sustains an adequate sensory function (Fettiplace and Hackney, 2006). In vertebrates, the inner ear is integrated by an osseous outer wall (otic capsule) and an internal membranous labyrinth (Ferrary et al., 2007). The two structures are separated by a liquid medium called perilymph which is presumed to be a derivate of blood plasma (Scheibe and Haupt, 1985) or cerebra-spinal fluid (Wangemann and Schacht, 1996). The membranous labyrinth itself is filled with endolymph, a solution with a unique ionic composition comparable to no other body fluid (Wangemann, 2006).

The role of the perilymph and endolymph in the inner ear can be condensed into three main functions. Physiologically, the inner ear fluids provide a unique ionic environment, which generates an electrochemical potential necessary for the adequate functioning of the auditory receptor cells (v. Békésy, 1952; Wangemann, 2006). Secondly, the inner ear fluids provides a homeostatic pressure equilibrium and serve as a propagation medium for sound-induced vibrations moving from the oval window to compliant basilar membrane locations (Duke and Jülicher, 2003; Reichenbach and Hudspeth, 2014; Robles and Ruggero, 2001; von Békésy, 1960). Finally, the inner ear fluids serve as a transport system for the distributions of nutrients and the removal of metabolic waste products from the inner ear (Lawrence, 1969).

The chemical composition of the inner ear fluids have been studied in a diversity of vertebrate taxa including fish (Enger, 1964; Ghanem et al., 2008; Payan et al., 1997; Rüsch and Thurm, 1989), birds (Runhaar et al., 1991; Sauer et al., 1999), amphibians (Bernard et al., 1986; Corey and Hudspeth, 1983; Valli et al., 1990), reptiles (Freeman et al., 1993; Ricci and Fettiplace, 1998) and mammals (Bosher and Warren, 1968; Couloigner et al., 1999; Flock, 1977; Johnstone et al., 1963; Makimoto et al., 1978; Silverstein, 1966; Silverstein and Schuknecht, 1966). Although the values reported for ions and proteins concentrations vary among the studies, the general agreement is that endolymph has a higher concentration of Potassium (K^+) than of Sodium (Na^+) and that perilymph has a higher concentration of Na^+ than of K^+ (Fernández, 1967; Peterson et al., 1978).

Chemically, the endolymph is rich in K^+ (approximately 150-180 mM) and Chlorine (Cl^-, approximately 150 mM), and low in Na^+ (approximately 1 mM). However the concentration of Na^+ and K^+ are similar among the different areas of the endolymphatic system (except for the endolymphatic sac in mammals, birds, reptiles and amphibians). Other components present in cochlear endolymph are Calcium (0.02 mM), Magnesium (0.01 mM), proteins (0.6 g/l) and glucose (<0.6 mM). In contrast, the perilymph has an ionic composition similar to that of extracellular fluids. The main cation is Na^+ (140 mM) and the anion is Cl^- (120 mM), while the concentration of proteins is low (~0.2 g/l). Unlike the endolymph system, the concentration of ions between the perilymph of the scala vestbuli and scala tympani are different, being more elevated in the scala tympani.

Contrary to vertebrates, in insects it is rare to find auditory organs immersed in a liquid medium. In general, insect auditory systems do not require the presence of a liquid environment for functioning. For instance, antennal ears respond directly to the particle velocity component of airborne sound through mechano-transduction (Jackson and Robert, 2006), while tympanal ears can have the transducers physically attached to the tympanum and operating in air (Bangert et al., 1998; Brechow and Sippel, 1985; Hoy and Robert, 1996; Oldfield, 1985). There are a few exceptions, however, in which the auditory organs are immersed in a liquid environment. This condition is observed in the ear of the green lacewing, *Chrysopa carnea* (Miller, 1970), in some aquatic hemipterans (Arntz, 1972), and in the bush-cricket *Copiphora gorgonensis* (Montealegre-Z et al., 2012). Thus, the ears of *C. gorgonensis*, and possibly

other bush-crickets represent a distinctive hearing system among insects by presenting a liquid-filled inner ear cavity similar to the vertebrate cochlea, known as the auditory vesicle (AV, Figure 4.1). Furthermore, the bush-cricket ear also has auditory mechanisms analogous to those observed in vertebrates, such as a lever system to amplify signals between the outer and inner ear (Montealegre-Z and Robert, 2015). Critically, it is likely that the chemical composition of the liquid contained in the AV provides specific conditions which will aid hearing sensitivity via various mechanisms, e.g. the tuning of the sensory cells, affecting travelling wave dispersal and velocity, or increasing reliable transduction of signals.

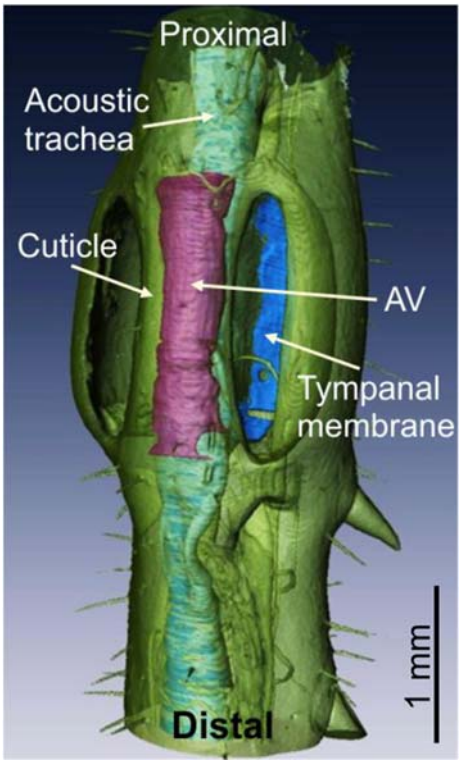

Figure 4.1. *Panacanthus pallicornis* tympanal ear µ-CT scan reconstruction. In this image the AV (purple) is located between the cuticle (green) and the acoustic trachea (light blue). 3D reconstruction: Fabio A. Sarria-S, µ-CT scan: Dr. Thorin Jonsson.

Copiphora gorgonensis is so far the only species in which it the presence of the AV has been reported, but it is believed that other species of Neotropical bush-cricket might possess a similar structure (Montealegre-Z and Robert, 2015), or at least that their hearing systems require a fluid medium for the dispersion of traveling waves.

4.2. Methods

4.2.1. Inner ear liquid extraction

For the chemical analysis of the liquid contained in the inner ear, seven species of bush-cricket were sampled (Figure 4.2.). Inner ear fluid was extracted from live specimens maintained in colonies as described in chapter 2, Specimens were placed on a platform made out of cork (5 cm x 2 cm), and gently restrained with staple clamps over the legs and the abdomen, while the front legs were held to the front by a brass wire (Figure 4.3). Once the insect was immobilised, the external cuticle of the tympanal slit was excised to gain visual and mechanical access to the tympanal membranes (Figure 4.3B), with the exception of *Phlugis poecila* whose intact tympanal slits allowed adequate access. The excisions were done using small pieces of stainless steel double edge razor blades placed in a blade holder.

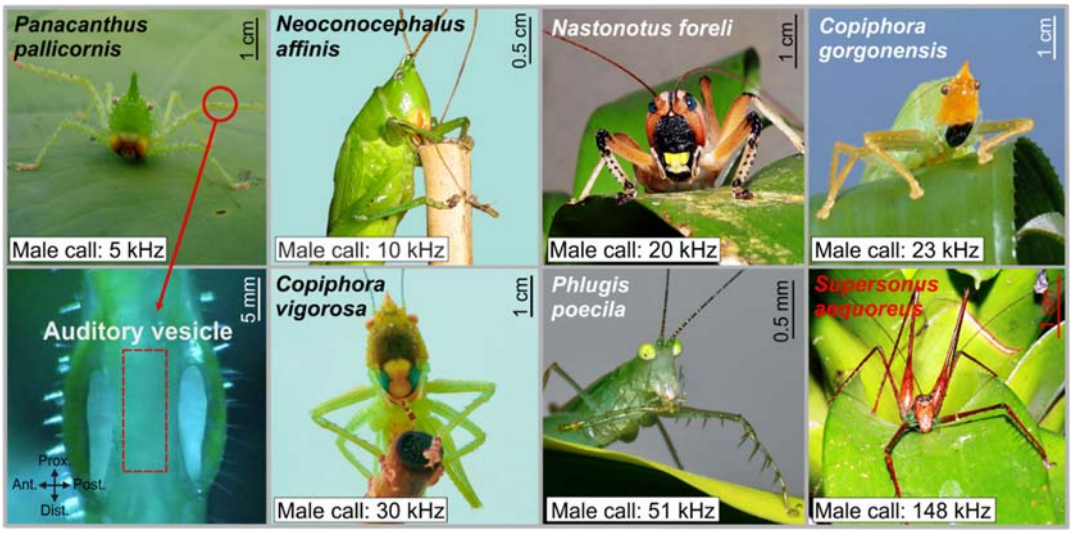

Figure 4.2. Studied species and their corresponding mating song peak frequency.

The liquid was extracted by capillarity action using glass microcapillaries, which were pulled from borosilicate glass tubing (external diameter: 1.0 mm, internal diameter: 0.8 mm; B120-69-8, Linton Instruments, Norfolk, England) using a micropipette puller (P30; Sutter Instruments, Novato, CA, USA) to produce tips with a width of ~10 µm. The microcapillary was mounted in a micromanipulator for precise movement of the tip through the tympanal plate into the fluid filled area beneath at the proposed position of the inner ear (Figure 4.1). With this method it was possible to extract samples of approximately 0.20 to 0.50 µl of liquid per ear. This technique also allowed the extraction of fluid without affecting the integrity of the auditory organ and reducing contamination from other body fluids. Extracted liquid volume was measured by injecting example samples into oil and measuring the diameter of the resultant spherical drop.

As a control, haemolymph samples were also extracted from the coxa of the hind leg of the same specimen employing the same procedure as with the

ears. The ear and haemolymph samples were deposited in a 0.5 ml polypropylene tube (Brand®, Sigma-Aldrich, Dorset, England) containing 20 µl of ethyl acetate (Sigma-Aldrich, Dorset, England). Ethyl acetate was used has a preservative solvent, since it does not react with the water contained in the samples. All the samples were kept in a freezer at approximately -20º C.

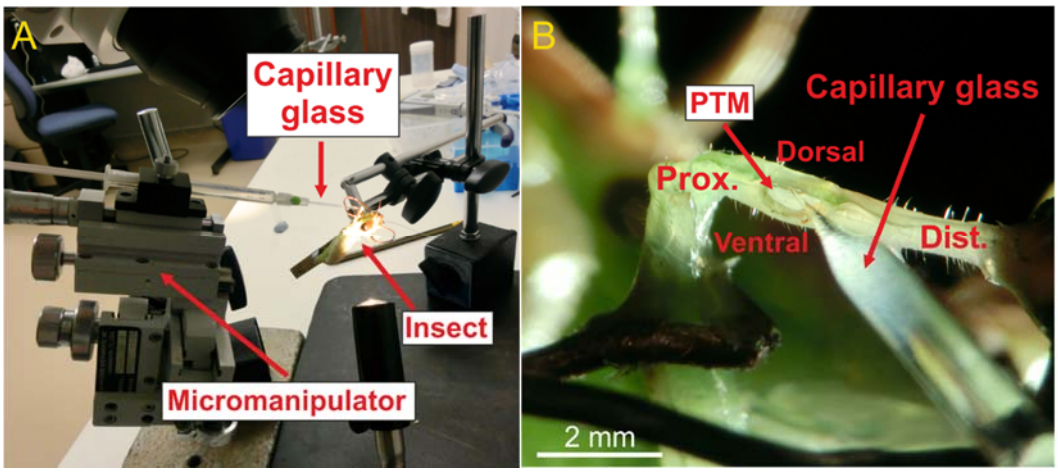

Figure 4.3. Mounting of specimen and close-up of the insect's ear. A) Specimen mounted for inner ear liquid extraction. B) Lateral view of the right leg. Once the posterior tympanal membrane (PTM) is exposed and the inner ear identified, the tip of the capillary glass is inserted through the tympanal plate. Proximal (Prox.) and Distal (Dist.).

4.2.2. Inductively coupled plasma optical emission spectrometry (ICP-OES)

ICP-OES was used as the analytical method for the study of the elemental composition of the inner ear liquid and hemolymph. This analytical technique is a type of emission spectroscopy that uses the inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element (Manning and Grow, 1997). This is accomplished by ionizing the sample with inductively coupled plasma

and then using the spectral emission of the different elements to identify and quantify the ions (Lara et al., 2005).

For the analysis an inductively coupled plasma-optical emission spectrometer Thermo Scientific™ iCAP™ 7000 ICP-OES Analyser (Thermo Scientific, UK) was used for quantitative/qualitative elemental analysis of the ear cavity samples. The ICP-OES was connected to CETAC® ASX260 auto sampler with 9 standard racks and two sets of sample trays each with 60 sample racks. All analyses were performed using axial mode as it offers a greater sensitivity at low concentrations. The following elements were selected for analysis using the specified wavelength in brackets [nm]: Lithium [670.784], sodium [589.592], potassium [766.490], magnesium [279.553], calcium [393.366], titanium [334.941], iron [259.940], phosphorus [177.495] and sulphur [180.731]. Elements like titanium were selected to assess any potential contamination as it is present in soil dust and other surfaces but unlikely to be found in biological samples. Samples were first diluted in 1 ml of deionised water, then vortexed for one minute to ensure a good mix. Following this procedure, the vortexed sample was added to a test tube with 3 ml of deionised water and loaded onto an autosampler tray (Figure 4.4). As a control, a reagent blank (only deionised water) was used in between samples and a multi-element standard solution, a periodic table mix certified reference material of elemental standards (Fluka Analytical,Sigma-Aldrich GmbH, Switzerland) was used to calibrate the molar mass of the ions present in the samples. Liquid flow rates were controlled with a peristaltic pump, with sample flow at 1 ml/min. A standard data acquisition run was implemented for the

analysis of 110 samples, with 3 replicates per sample. The plasma used was generated using a flow of argon gas. Data were acquired using the software supplied by the manufacturer.

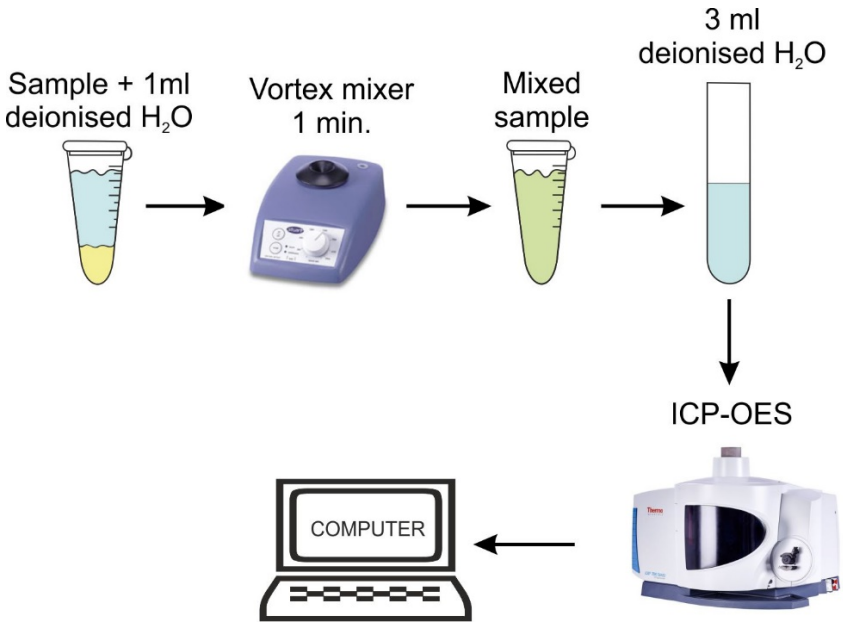

Figure 4.4. Schematic representation of the ICP-OES analysis protocol for the inner ear liquid samples.

4.2.3. Data analysis

After all the raw data was collected, a preliminary data treatment was necessary for adjusting the data from different species. For this, the volume of 3D reconstructions of μ-CT scans of the ear cavity (Figure 4.1) were used for the adjustment. Therefore, the initial values obtained with the ICP-OES technique were calibrated using a reference multi-element standard solution. With this procedure it is was determined the number of moles of each element present in the samples. To compare quantitatively the values from different species, it was necessary to calculate the ionic concentration (molarity) in the ear liquid samples. For this, the moles from the calibration step were divided

by the auditory vesicle geometric volume (in litres), assuming that this represents the volume of the ear solution. As a result, the elemental concentration was compared in milimoles per litre (mmol/l). Additionally, the concentration values from only deionised water (blanks) were subtracted from the samples because they represented values added by the solvent and by contamination. Sample contamination is imposed by different sources in the laboratory, among them, the need of diluting sample solutions prior to the analysis and by the equipment itself. This estimation allowed the identification of those ions with very low concentrations (close to zero) that appeared to not be contributing to the chemical profile of the samples and consequently were not used for the rest of the analysis.

A graphical representation of the median values and standard errors were generated for ion concentration of hemolymph and inner ear liquid samples. An independent Mann-Whitney *U*-test was applied to test the difference between hemolymph and inner ear liquid median concentration values per species. To compare the median ion concentration among species, a Kruskal-Wallis test was performed. The relationship between male courtship song peak frequency and ear liquid and hemolymph total ion concentration was tested with a generalized linear mix model (GLMM). In both models, song frequency was set as a fixed factor with individual bush-cricket and sample volume as random effects to control for species-differences in samples quantity. The statistical analyses were performed by using SPSS v. 22 (IBM, 2013), and the lme4 package (Bates et al., 2014) run in R v. 3.3.1 (R Development Core

Team, 2016). All P values were two-sided and values less than 0.05 were considered to indicate statistical significance.

4.3. Results

4.3.1. Ionic composition of the hemolymph

A multi-element trace analysis by ICP-OES was run for 16 elements (Li, Na, K, Mg, Ca, Si, Ti, Fe, Co, Ni, Cu, Zn, P, S, Sr, and Cd) for the inner ear liquid of seven species and the hemolymph of six of them. The ionic composition of hemolymph is variable among insects, but sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) are the most prominent cations of the dissolved inorganic elements (Clark and Craig, 1953; Nation, 2008). Therefore, these cations were chosen as focus elements with the purpose of comparing our results with those previously published for hemolymph chemical composition in Arthropoda (Jeuniaux, 1971). The mean concentration values and their respective range are summarised in Table 4.1.

Table 4.1. Ion mean concentration values (mmol/l) for Na^+, K^+, Mg^{2+}, and Ca^{2+} in the hemolymph.

Ion	Species	N	Mean (mmol/l)	Std. Deviation (mmol/l)	Std. Error (mmol/l)	Minimum (mmol/l)	Maximum (mmol/l)
	C. vigorosa	11	60.91	44.97	13.56	5.24	150.87
	C. gorgonensis	7	56.29	40.80	15.42	13.71	120.49
	N. foreli	8	43.50	35.11	12.41	17.17	123.13
Na^+	*P. pallicornis*	6	53.86	60.42	24.66	0.49	168.04
	N. affinis	4	23.34	18.55	9.27	6.65	44.73
	P. poecila	3	38.06	16.56	9.56	26.73	57.06
	S. aequoreus	2	3.07	0.30	0.21	2.86	3.28
	C. vigorosa	11	48.14	55.01	16.59	0.34	169.40
	C. gorgonensis	7	49.82	91.80	34.70	3.82	256.22
	N. foreli	8	10.15	11.55	4.08	2.99	35.06
K^+	*P. pallicornis*	6	9.42	9.36	3.82	0.03	26.77
	N. affinis	4	12.29	9.19	4.59	3.81	24.45
	P. poecila	3	6.44	5.44	3.14	2.97	12.71
	S. aequoreus	2	0.44	0.40	0.29	0.16	0.73
	C. vigorosa	11	10.26	10.93	3.29	0.26	32.95
	C. gorgonensis	7	14.49	15.70	5.94	1.45	40.75
	N. foreli	8	15.91	14.94	5.28	2.49	35.32
Mg^{2+}	*P. pallicornis*	6	24.66	26.44	10.80	0.01	74.19
	N. affinis	4	10.77	7.95	3.97	0.14	18.65
	P. poecila	3	1.58	1.30	0.75	0.44	2.99
	S. aequoreus	2	0.11	0.04	0.03	0.08	0.14
	C. vigorosa	11	15.55	11.67	3.52	0.00	35.88
	C. gorgonensis	7	3.74	6.85	2.59	0.00	17.40
	N. foreli	8	20.67	35.61	12.59	0.00	105.99
Ca^{2+}	*P. pallicornis*	6	6.29	9.05	3.69	0.00	19.12
	N. affinis	4	3.07	3.87	1.93	0.00	8.03
	P. poecila	3	5.35	0.56	0.33	4.73	5.84
	S. aequoreus	2	3.12	0.11	0.08	3.05	3.20

4.3.2. Ionic composition of the inner ear liquid

The mean concentration values of Na^+, K^+, Mg^{2+}, and Ca^{2+} for the inner ear liquid are summarised in Table 4.2.

Table 4.2. Ion mean concentration values (mmol/l) for Na^+, K^+, Mg^{2+}, and Ca^{2+} in the inner ear liquid.

		N	Mean (mmol/l)	Std. Deviation (mmol/l)	Std. Error (mmol/l)	Minimum (mmol/l)	Maximum (mmol/l)
Na^+	*C. vigorosa*	11	44.78	42.98	12.96	8.95	111.4
	C. gorgonensis	7	22.00	17.29	6.53	2.24	54.51
	Na. foreli	6	50.48	37.27	15.22	12.79	102.33
	Ne. affinis	8	87.58	52.68	18.62	18.23	145.21
	P. pallicornis	4	48.72	35.39	17.69	1.85	77.76
	Ph. Poecila	4	83.34	74.49	37.25	18.39	151.57
	S. aequoreous	2	10.68	1.45	1.02	9.66	11.71
K^+	*C. vigorosa*	11	7.24	8.14	2.45	0.04	29.01
	C. gorgonensis	7	16.31	24.15	9.13	0.16	67.66
	Na. foreli	6	23.18	27.36	11.17	0.24	71.29
	Ne. affinis	8	3.40	3.90	1.38	0.11	11.12
	P. pallicornis	4	74.31	72.96	36.48	-0.64	172.76
	Ph. Poecila	4	1.24	0.78	0.39	0.39	1.95
	S. aequoreous	2	0.63	0.27	0.19	0.44	0.82
Mg^{2+}	*C. vigorosa*	11	1.41	1.21	0.36	0.071	3.79
	C. gorgonensis	7	23.96	37.46	14.16	0.092	87.70
	Na. foreli	6	24.45	33.92	13.85	0.094	77.47
	Ne. affinis	8	18.60	33.19	11.74	0.036	72.53
	P. pallicornis	4	25.161	13.28	6.64	13.54	44.25
	Ph. Poecila	4	0.38	0.32	0.16	0.10	0.75
	S. aequoreous	2	0.33	0.01	0.01	0.32	0.34
Ca^{2+}	*C. vigorosa*	11	0.00	0.00	0.00	0.00	0.00
	C. gorgonensis	7	2.43	6.43	2.43	0.00	17.01
	Na. foreli	6	0.00	0.00	0.00	0.00	0.00
	Ne. affinis	8	0.00	0.00	0.00	0.00	0.00
	P. pallicornis	4	0.1	0.24	0.12	0.00	0.50
	Ph. Poecila	4	0.00	0.00	0.00	0.00	0.00
	S. aequoreous	2	0.00	0.00	0.00	0.00	0.00

4.3.3. Inner ear liquid ion concentration among species

A comparison to evaluate pairwise differences among species ion mean rank concentration were conducted with the use of a Kruskal-Wallis test (Figure 4.5). There was not a stastically significant difference among species regarding Na^+ ($H = 10.82$, $df = 6$, $p = 0.094$), K^+ ($H = 9.63$, $df = 6$, $p = 0.141$), and Mg^{2+} ($H = 11.21$, $df = 6$, $p = 0.082$). There was a statistically significant

difference for Ca^{2+} mean rank concentration values (H = 13.16, df = 6, *p* = 0.041).

A post-Hoc comparison to evaluate pairwise differences among species Ca^{2+} mean rank concentration were conducted with the use of a Mann-Whitney *U* test after applying a Bonferroni adjustment. The tests indicated that there is a statistically significant difference between *P. panacanthus* and *C. vigorosa* (*U* = -10.25 *p* = 0.028), and *P. panacanthus* and *Neo. affinis* (*U* = -10.25 *p* = 0.047).

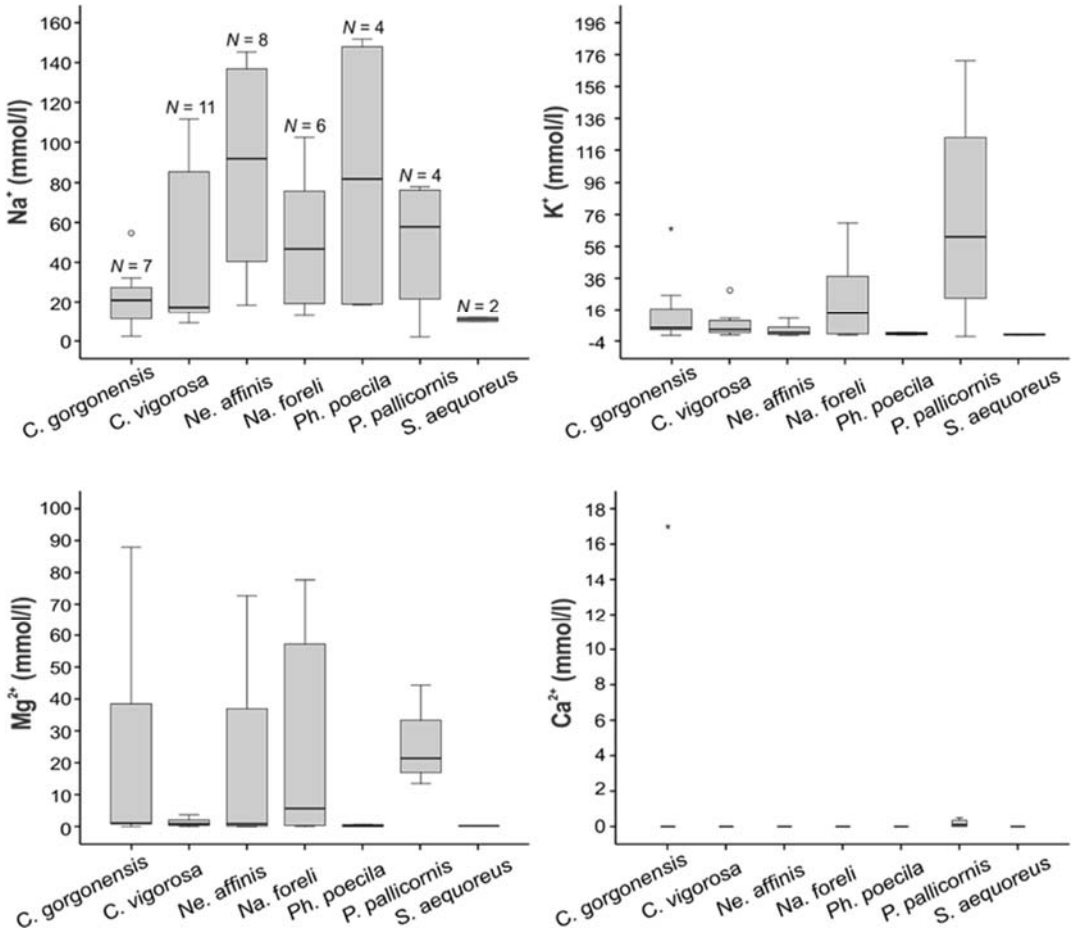

Figure 4.5. Inner ear liquid ion concentration median and quartiles values in mmol/l (± s.e.) for the seven studied species.

4.3.4. Hemolymph *vs* inner ear liquid

Figure 4.6 shows that Na^+ is the cation with the highest median concentration values in both, hemolymph and inner ear liquid, follow by K^+ and Mg^{2+}. This quantitative distribution is comparable to what has been reported in previous studies, but a notable feature is the very low concentration to non-appearance of Ca^{2+} in the inner ear liquid. The appearance of Ca^{2+} in some of the samples (Figure 4.6B and Figure 4.6E), can be attributed to hemolymph contamination. It is probable that during the extraction of inner ear liquid, the piercing of the tympanal plate with the glass capillary suddenly changed the inner ear's pressure, thus withdrawing hemolymph from the surrounding tissue.

Additionally, a Mann-Whitney *U*-test was performed to compare the median ion concentration between the hemolymph and inner ear liquid per species (Table 4.3) and there was a significant difference in most of the species for Ca^{2+}.

Table 4.3. Mann-Whitney *U*-test for ion median concentration values in hemolymph and inner ear liquid per species.

species	N	Na^+	K^+	Mg^{2+}	Ca^{2+}
C. vigorosa	Liquid: 11 Hemolymph: 11	U = 48; *p* = 0.41	U = 32; *p* = 0.06	U = 32; *p* = 0.06	U = 5.5; *p* = 0.001
C. gorgonensis	Liquid: 7 Hemolymph: 7	U = 9; *p* = 0.048	U = 16; *p* = 0.28	U = 18; *p* = 0.41	U = 21; *p* = 0.53
Ne. affinis	Liquid: 8 Hemolymph: 8	U = 17; *p* = 0.11	U = 12; *p* = 0.04	U = 16; *p* = 0.09	U = 8; *p* = 0.004
N. foreli	Liquid: 6 Hemolymph: 6	U = 16; *p* = 0.75	U = 13; *p* = 0.42	U = 15; *p* = 0.63	U = 9; *p* = 0.06
P. pallicornis	Liquid: 4 Hemolymph: 4	U = 5; *p* = 0.39	U = 4; *p* = 0.25	U = 2; *p* = 0.08	U = 6; *p* = 0.54
Ph. poecila	Liquid: 4 Hemolymph: 3	U = 6; *p* = 1	U = 0; *p* = 0.03	U = 2; *p* = 0.16	U = 0; *p* = 0.02

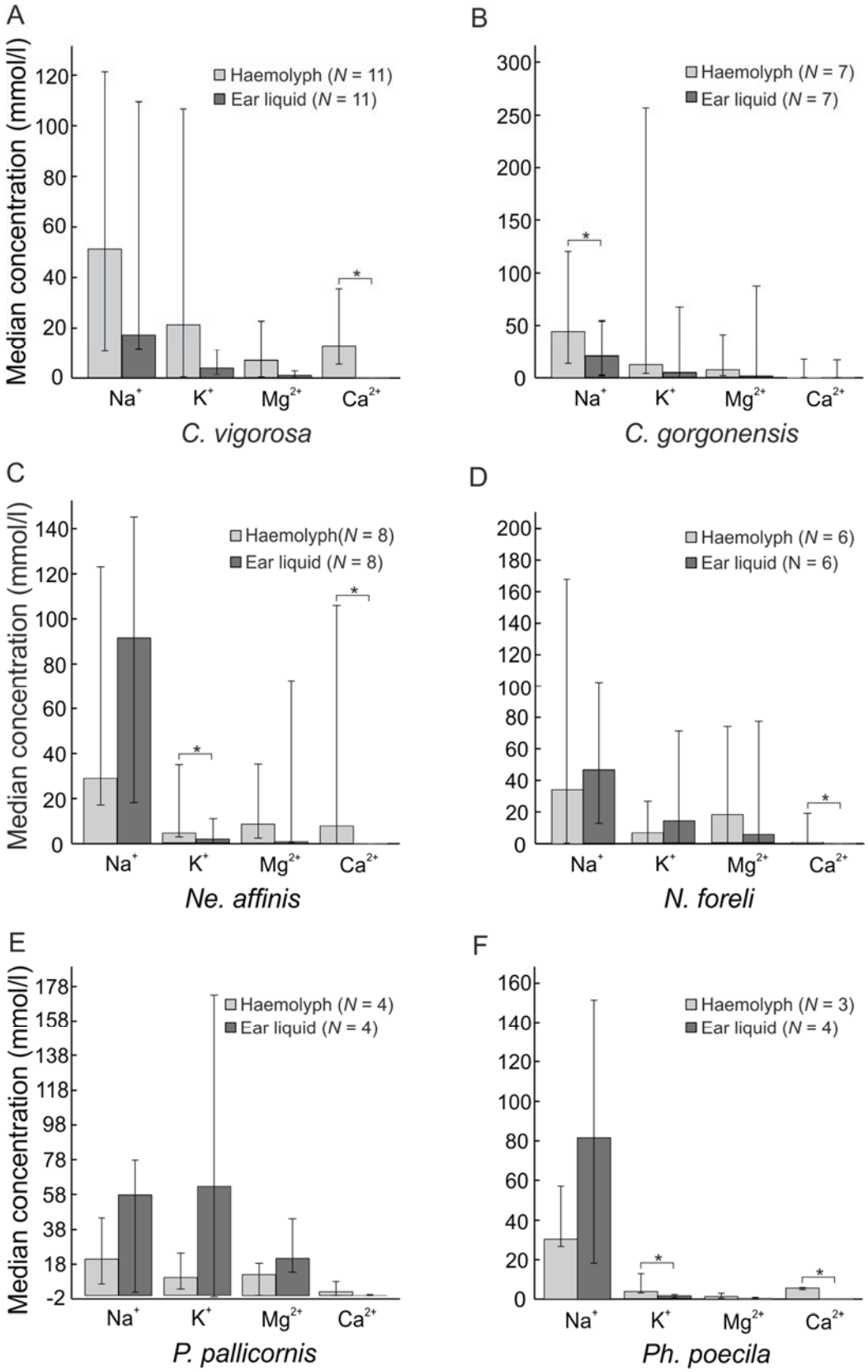

Figure 4.6. Comparison between hemolymph and inner ear liquid mean concentration values in mmol/l (± s.e.) for the studied species, except *S. aequoreus*.

4.3.5. Ca^{2+} in the inner ear liquid

Calcium is perhaps the most influential polyvalent ion found in living cells (Clark and Craig, 1953). In insects, Ca^{2+} is involved in a diversity of physiological processes, e.g., it participates in intermediary metabolic processes, in locomotion, or in neural signalling (Nation, 2008). In view of the extremely low Ca^{2+} mean values obtained with the ICP-OES analysis for the liquid samples (Table 4.2), the Mann-Whitney *U*-test showed that in four species the inner ear liquid Ca^{2+} median values were significantly different from those obtained for hemolymph samples (Table 4.3), while, three species, *C. gorgonensis*, *N. foreli*, and *P. pallicornis* presented values that were not significantly different (Table 4.3).

4.3.6. Total ion concentration and mating song carrier frequency

The relationship between total ion concentration (ear liquid and hemolymph) and male song carrier frequency (Figure 4.7) was tested with a general linear mix model (GLMM), in which frequency was set as a fixed factor, with individual bush-cricket and sample volume as random effects. The GLMM model found that the total ion concentration of ear liquid and hemolymph samples were not significantly related to the male song carrier frequency (Ear liquid: $\beta \pm$ s. e. = - 0.03 ± 0.01, T_1 = -23.40, p = 0.25; Hemolymph: $\beta \pm$ s. e. = - 0.005 ± 0.01, T_1 = -0.61, p = 0.65).

Additionally, a comparison to evaluate pairwise differences among species total ion concentration were evaluated with a Kruskal-Wallis test. The test showed that there was not a stastically significant difference among species

for the ear liquid (H = 11.87, df = 6, p = 0.065), and hemolymph samples (H = 8.43, df = 6, p = 0.21).

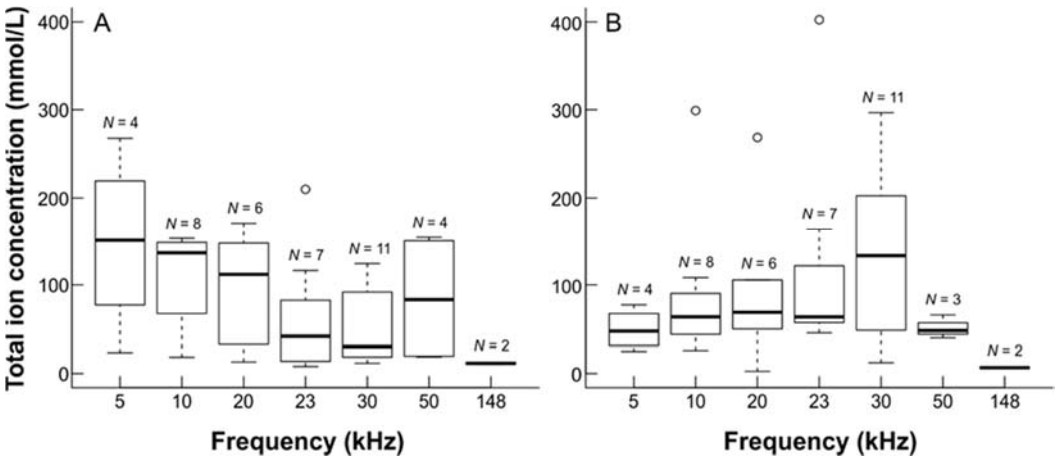

Figure 4.7. Total ion concentration and carrier frequency of male song. A) Inner ear liquid combined ion values (Na^+, K^+, Mg^{2+}, and Ca^{2+}). B) Hemolymph combined ion values (Na^+, K^+, Mg^{2+}, and Ca^{2+}). Bars represent standard errors with a coefficient interval of 95%.

4.4. Discussion

4.4.1. Ion concentration

In Orthoptera, the concentration of inorganic cations in the hemolymph fluctuates strongly within the taxa and it is also dependent of the developmental stage (Duchateau et al., 1953). Large differences in concentration of the hemolymph from a diversity of species have been reported. Additionally, there is great variability in results obtained even for a single species. Therefore, it is difficult to make comparisons across species from the available data. Subsequently the variability could be related to differences in techniques and not necessarily to differences between species. Although, there is not an established reference range for Na^+, K^+, Mg^{2+}, and Ca^{2+} concentration values, it has been considered that for this taxonomic

group there is a tendency for a relatively high Na^+ : K^+ ratio, while the Mg^{2+} : Ca^{2+} ratio is about 1 (Sutcliffe, 1963). This condition is believed to represent an early evolutionary state (Jeuniaux, 1971). Although, the hemolymph concentration mean values for Na^+, K^+, Mg^{2+}, Ca^{2+} obtained for the six species used in this analysis (Table 4.1), do not meet the tendency suggested for the order Orthoptera (Jeuniaux, 1971), although, in Figure 4.6, it is perceived a tendency for a major concentration of ions of Na^+ and K^+, while mean values for Mg^{2+} and Ca^{2+} are close to the range of the published data (Duchateau et al., 1953; Florkin and Jeuniaux, 1974; Nation, 2008).

The obtained analytical results confirm that the ion composition of the inner ear liquid and hemolymph samples is mainly composed by Na^+, K^+, Mg^{2+}, and Ca^{2+} (Table 4.1 and Table 4.2), however, their concentration varies between the two solutions (Figure 4.6). A factor contributing to the variation between hemolymph and inner ear liquid concentration is the binding of ions to macromolecules as such as proteins, carbohydrates, or nucleic acids (Weidler and Sieck, 1977). It was observed in *Periplaneta americana* that hemolymph sampled from different body parts of the same specimen showed an unequal distribution of Na^+, K^+ and Ca^{2+} (Pichon, 1970). Since inorganic ions circulate rather without restrictions in an aqueous medium, it is likely that the binding of slowly diffusing macromolecules in the hemolymph to inorganic ions enhance their distribution (Weidler and Sieck, 1977). In the case of bush-crickets with a fluid-filled cavity or acoustic vesicle, contained liquid is separated from the hemolymph circulating through the tibia (Montealegre-Z et al., 2012). This physical isolation potentially filters out particles and determines the presence

of certain macromolecules in the inner ear liquid and consequently the local concentrations of ions. In the hemolymph, Ca^{2+} is associated with proteins, derived proteins, and other organic compounds (Clark and Craig, 1953), while its absence in most of the inner ear liquid samples (Table 4.2), could be explained by the unavailability of those organic compounds in the inner ear. Low concentration of Ca^{2+} is also common feature in the mammalian cochlear fluids, with Ca^{2+} concentration values in the endolymph of 0.02 mM, and 0.2 mM for the perilymph (Ferrary et al., 2007; Wangemann, 2006), whereas in the serum on blood plasma Ca^{2+} concentration is around 1.16 mM/l (Moore, 1970)

4.4.2. Inner ear liquid function

The main purpose of ICP-OES analysis was to evaluate if the liquid covering the auditory organ was hemolymph, hence the hemolymph channel runs along the dorsal surface of the acoustic trachea. The fact that the ear liquid and the hemolymph presented small but consistent differences (Figure 4.6), these are not strong evidence to assume that they are different (Table 4.3). Additionally, as it was mentioned above, ion concentration in the hemolymph varies between body regions of the same individual (Pichon, 1970). Even though, ion concentration varies among bodies part, low concentration of Ca^{2+} in the ear samples was a common feature (Table 4.2). One could ask, if Ca^{2+} plays an important role in signal transduction pathways, where they act as a second messenger for neurotransmitter release in neurons (Berridge, 1998; Neher and Sakaba, 2008), how a low concentration helps in the hearing process? To answer this, it is necessary to look at the mammalian inner ear. Despite the

fact that Ca^{2+} participates in the transmission of neural signal in other body parts, in the inner ear its function is to intervene in homeostatic and modulation of transduction mechanism rather than in a mechanoelectrical transduction process (Wangemann and Schacht, 1996).

Independently of a physiological function, from a mechanical point of view, perilymph and endolymph have practically identical properties. The inner ear as a whole employs its hydrodynamic effects in combination with local micromechanical features of the basilar membrane, organ of Corti, and hair cells to spatially separate the different frequency components of sound (Reichenbach and Hudspeth, 2014). In the same manner, in bush-crickets the low Ca^{2+} and the containment of the liquid in a vesicle (Figure 4.1) might facilitate mechanical processes in the ear. With the existing information, it can be assumed that the function of the bush-cricket ear liquid is mechanical and it might provide an optimal osmotic environment for the sensory cells.

4.4.3. Male calling song peak frequency and ion concentration

Even though there was not a statistically significant relationship between the male courtship song peak frequency and the total ion concentration of the inner ear liquid (Figure 4.7A), a specific concentration of ions might be an additional factor enhancing the tuning of bush-cricket ear to their conspecific calling song peak frequency. Density and viscosity of the liquid environment are determined by the ion concentration. These two properties are key factors for the transmission of acoustic signals, in other words, according to Newton-

Laplace equation (Fletcher, 2007), sound velocity is dependent of these two properties, equation 4.1.

$$c_v = \sqrt{\frac{k}{\rho}} \tag{4.1}$$

Where c_v is the sound velocity, k is the bulk modulus of the dispersive medium and ρ is the density of the medium. Therefore, acoustic signals propagating in a fluid as longitudinal pressure waves and are affected by these physical properties. Interestingly, in the mammalian cochlea sound signals propagate as transversal waves on the elastic basilar membrane with a much smaller wavelength and velocity than a sound wave moving through a fluid (Reichenbach and Hudspeth, 2014; Warren et al., 2016). In chapter 3, the auditory response in the ear of *P. poecila* was characterised as a travelling wave based in the calculated phase lags, velocities and wavelengths (Figure 3.7 and Figure 3.8). Subsequently, these travelling waves are a type of transversal wave (Elliott and Shera, 2012; Patuzzi, 1996), and their propagation velocities are affected by the sound frequency and not by the medium's density, and viscosity (Dallos, 1992).

A species-specific ion concentration could be related to the tuning of the hearing organ to the male calling song peak frequency. If the crista acustica is considered as a mass-spring damped system (Babbs, 2011). According to the harmonic oscillator's natural resonance equation (Patuzzi, 1996):

$$\omega_n = \sqrt{\frac{k}{m}} \quad (4.2)$$

The scolopidium natural resonant frequency (ω_n) could be determined by its mass (*m*) and spring constant (*k*), whereas, under a damping condition the damped natural frequency (ω_d, equation 4.3) would be affected by the damping effect (Fletcher, 1992).

$$\omega_d = \omega_{n\sqrt{1-\zeta^2}} \quad (4.3)$$

The damping ratio (ζ) is provided by the Inner ear liquid, which is intrinsic to its ion concentration. The value of the damping ratio ζ defines the behaviour of the system. A damped harmonic oscillator can be overdamped (ζ > 1), critically damped (ζ = 1), or underdamped (ζ < 1). In the two first conditions the system does not oscillate while an underdamp system oscillates with the amplitude gradually decreasing to zero (Blake, 2010).

Under these circumstances species with hearing response in the audible spectrum possibly improve their tuning to low frequencies by having an inner ear liquid with higher damping effect while species with auditory perception in the ultrasonic range benefit from low viscous damping coefficients. For instance, in Figure 4.6A, it is seen that ion concentration tends to decrease in species with high frequencies, except for the ion concentration of *C. vigorosa* and *Ph. Poecila*. In this two species the male song peak frequency is 29 kHz and 51 kHz respectively (Figure 4.1) and the calls are characterised for a broad band spectrum (Figure A1.1 and Figure A1.2C). Consequently, the ion

concentration in these two species might be associated with the need of having the ears tune to a wide range of frequencies. The biomechanical effect of the inner ear liquid ion concentration remains elusive, but a future approach would be the characterization of its viscoelastic and damping properties and their relationship with scolopidium resonant response.

4.4.4. Inner ear liquid effect on LDV measurements

The non-invasive measurement of auditory activity in bush-crickets is possible in those species with certain levels of cuticle transparency by using laser Doppler vibrometry as it was demonstrated in chapter 2. Although the refractive index of the cuticle nor the inner ear liquid have not been measured, it is possible that this property has an effect on the LDV measurements. The presence of a liquid medium between the ear's protective cuticle and the crista acustica, reduces the laser beam scattering by providing a refractive index-matching effect and improving the laser response (Vargas et al., 1999). Data regarding the refractive index of insect hemolymph are scarce, but it has being reported to be higher than the refractive index of the water (Miyajima, 1982). Even though the inner ear liquid and hemolymph chemical composition are not the same, it could be assumed that the inner ear liquid refractive index might have a value close to that estimated for the hemolymph. Thus, a high refractive index might increase the resolving power between the cuticle and the CA as occurs with the use of immersion oils in light microscopy (Cargille, 1985). Finally, the inner ear concave surface (Hummel et al., 2017) combined with the refractive index of the liquid, together have an optical effect comparable to a plano-convex lens. As a consequence, this feature increases the numerical

aperture of the laser beam while reducing the characteristic irradiance loss of a Gaussian beam (Duocastella et al., 2015).

Chapter 5: General Discussion

Living organisms through time have evolved morphological structures, metabolic processes, and behavioural traits in response to the physical and ecological pressures imposed by the environment. Under this context, different mechanisms of communication were described in the introductory chapter, with an emphasis on acoustic communication in land dwelling vertebrates and insects. As stated in the introduction, from all the different communication systems (e.g. visual, chemical, or mechanical), the use of sound is the most widespread among animals. Within this it is possible to appreciate different levels of anatomical and behavioural adaptations, from a single mechanosensory cell to sophisticated hearing systems, or from startle responses to sound driven navigation systems. For instance, insects and vertebrates have evolved sensitive hearing multiple times, each with their unique manifestations, but with same need of transducing acoustic energy into electrical signals using specialized structures that function under the same biophysical principles. Consequently, functional properties have been investigated and described for a variety of auditory systems in vertebrates (Nobili et al., 1998) as well as analogous mechanisms in insects (Albert and Kozlov, 2016; Stumpner and von Helversen, 2001).

Regardless of all the advances in the field of hearing research, processes such as active amplification, travelling waves, and frequency discrimination remain unclear. Taking advantage of the anatomical and functional features of bush-cricket ears, these insects could represent an alternative approach to tackle the experimental limitations found in vertebrates (Sarria-S et al., 2017).

Therefore, the aim of this thesis was to evaluate the suitability of bush-crickets for non-invasive experimentation *in-vivo*, and to provide a preliminary description of the elemental composition of the liquid contained in the bush-cricket inner ear. This approach expands our knowledge of complex hearing processes, which cannot be measured in the mammalian inner ear, and opens new paths for research in hearing.

5.1. Cuticle transparency

In chapter 2, it was demonstrated that transparent cuticle effectively supports the visualization and measurement of the auditory activity with minimum to practically no manipulation at all of the hearing organ. The main advantage of this approach is that it overcomes the need for surgical intervention (i.e. removing the cuticle). Additionally, the ability to image through the cuticle provides an unprecedented opportunity for experimental manipulation such as the use of voltage-sensitive dyes to follow neuronal activity of the mechano-sensory cells involved in the hearing process in real time (Baden and Hedwig, 2010; Isaacson and Hedwig, 2017; Nikitin et al., 2015). From the point of view of experimental protocols, this method and the use of invertebrates, and especially insects, has proved to be logistically practical, economical, and less unpopular regarding aspects of animal welfare (Guhad, 2005; Scharrer, 1987). Furthermore, this type of comparative study provides insights on the evolution of acoustic perception, and with improvements it could also lead to the finding of new principles that might not be discovered through mammalian research alone.

5.2. Travelling waves

Travelling waves were proposed by Georg von Békésy to explain the mechanism of frequency analysis, but their principles are not completely understood and in fact it is not clear if they exist. Two hypotheses have been proposed to explain the travelling wave phenomena in the mammalian cochlea: 1) The mechanical anisotropy of the basilar membrane (von Békésy 1960), and 2) The individual resonances of hair cells (Wilson 1992). Thus far, defendants of each hypothesis rely mostly on mathematical models to approach the problem. An easy-to-access cochlea is ideal to approach this problem, and bush-crickets provide a good alternative. The bush-cricket auditory organ being small (<1mm) is not coiled, and lies immediately below the cuticle on the foretibia, which is in many cases transparent, allowing internal access with micro-scanning lasers as shown in chapter 3.

A key application of such studies would be the investigation of the mechanical origin of the travelling wave observed in the cochlea, and currently two hypotheses have been proposed to explain this phenomenon. Firstly, that travelling waves arise from anisotropic properties of the basilar membrane, resulting in tonotopically arranged displacement maxima causing excitation of the sensory cells (Robles and Ruggero, 2001). And secondly, that the observed travelling wave is a by-product of independently resonating sensory cells, coupled by a tectorial membrane (Bell, 2012b). Therefore, wave dispersion could be influenced by many factors, such as the nature of elastic coupling, geometry, and frequency, as well as by variations in physical properties. Only novel experimental designs, such as presented here, may

open avenues of research which help answer such fundamental questions in auditory mechanics.

5.3. Inner ear ion composition

Based on the obtained analytical chemistry results in chapter 4, it can be assumed that the ionic composition of the inner ear liquid is composed of the same ions that have been reported in the hemolymph, however, the ion concentrations between the two fluids is different and the absence of Ca^{2+} is a noticeable feature in the inner ear liquid. Although, the analytical technique chosen for the analysis provided information regarding the elemental composition, a more detailed chemical characterization is still required for the identification of other compounds or macromolecules such as proteins, carbohydrates, or lipids. A more comprehensive description of the ear liquid could shed light on the lipidic nature suggested by Montaealegre-Z et al. (2012), taking into account that a lipid filled cavity has been reported for the ear of the tree weta *Hemideina* sp. (Lomas et al., 2012).

5.4. Inner ear liquid compartmentalisation and biomechanical effects

In insects, the hemolymph ion concentration varies significantly among individuals and also among the different body parts of the same individual (Pichon, 1970). Ion concentration is affected by diet, water content, and the heterogeneous flow of the hemolymph trough the body (Jeuniaux, 1971; Weidler and Sieck, 1977). If the inner ear liquid is considered as being a continuation of the hemolymph channel, its ionic concentration could be susceptible to fluctuations, just as occurs in other body regions. If this is the

case, sudden variations in ion content might have negative effects on the auditory response by increasing or reducing its sensitivity. In humans, there are numerous hearing disorders that are the direct result of disturbed ion homeostasis (Anniko and Wróblewski, 1986; Trune, 2010). For instance, K^+ intoxication of the perilymph produces pathological disorders of hair cell activity and alterations in cochlear micromechanics (Zenner et al., 1994). Clinical cases of hearing loss, tinnitus, and Meniere's attacks, are possibly triggered by leakage of endolymph (Flock and Flock, 2003; Kingma and Wit, 2010). Therefore, a constant ion concentration in bush-crickets can be reached by means of physical separation of the inner ear liquid from the hemolymph.

From the biomechanical perspective, the containment of the inner ear liquid effectively makes it an incompressible fluid. Thus, this feature has an effect on the movement on the hearing structures imbedded in the liquid. A similar condition takes place in the inner ear of mammals, where the cochlear fluid are restricted by the cochlea's osseous walls (Lighthill, 1991). In addition, endolymph and perilymph pressures are maintained equally (Long III and Morizono, 1987; Takeuchi et al., 1990) by highly compliant membranes bounding the endolymphatic space (Wit et al., 2000). It is believed that the incompressibility of the perilymph is crucial for the mechanical movement of the basilar membrane and the opening of ion channels of the outer hair cells (Salt et al., 2009). The fluid mass affects the dynamics of the basilar membrane, loading its different parts by amounts that depend upon the local wavelength (Nobili et al., 1998; Ramamoorthy et al., 2010). Another approach

is that under this condition the pressure transferred by the ossicles of the middle ear is transmitted simultaneously to sensory cells (Bell, 2012a). In bush-crickets, comparable conditions are also observed, the rigid enclosure is provided by the dorsal cuticle and surrounding structure such as the tympanal membranes, acoustic trachea, and the colloidal material at each end of the inner ear cavity (Montealegre-Z et al., 2012).

A stable viscosity is another advantage of the liquid isolation. In liquids, this physical property is determined by the substances dissolved in it (Welty and Gelhar, 1991). In fluid dynamics, viscosity is a property that affects the fluid propagation speed and drag of immersed objects. Being the latter is essential for the transduction of mechanical stimuli to neural signals in mammals. Traveling waves in the cochlea are based on fluid inertia and outer hair cell stiffness: if there is a viscosity increase in the fluid, the cochlea's mechanical response and wave pattern is altered. Studies on cochlear models show that an increase in fluid viscosity causes a decrease in basilar membrane motion (Tonndorf, 1957), and the sensitivity is thus reduced (Gan et al., 2007). In another model, fluid viscosity was needed to suppress standing waves (White and Grosh, 2005).

As it was mentioned on chapter 3, Hummel et al. (2016) demonstrated that the activation of the auditory transducer channels of the bush cricket *Mecopoda elongata* is based on a mechanical sound-induced motion. The spatial phase changes in the mechanical motion induce a tilt of the cap cell and the stretching of the scolopidium neuron. For this mechanical tilt to be possible, it

is necessary that the applied force by the motion of the travelling wave overpasses the drag generated by the cap cell. This resistance is determined by the cap cell mass, surface area, and the viscosity of the liquid.

5.5. Future directions

It has been demonstrated from the molecular (Albert and Kozlov, 2016; Göpfert and Hennig, 2016), and the biomechanical (Montealegre-Z and Robert, 2015) perspectives that vertebrates and insects have, in their unique ways, embraced analogous hearing structures and processes to perceive sound. Thus, supplementary investigations of the chemical composition of the inner ear liquid in non-invertebrate models are imperative to contribute to this increasing field of hearing research. For instance, experimental data could be obtained by evaluating the effects of a change in a particular ion concentration on the activity of the sensory cells and the whole auditory response. In bush-crickets this could be achieved by taking advantage of the accessible ear. Therefore, a method for altering the concentration of a specific ion may be a modification of the protocol described in chapter 4 for the sampling of the ear liquid, but instead of drawing samples, it could be used to inject fluids to adjust and reach a desirable ion concentration.

Another feature that needs to be considered for future investigation is the regulation of the concentration and flow of inorganic ions in the inner ear liquid. In mammals homeostasis of the cochlear fluids is regulated by biochemical and biophysical processes, e.g. energy-dependent cation pumps, membranes, or cell tight junctions (Juhn, 1973; Wangemann and Schacht, 1996). Thus, in

bush-crickets, the observed differences in composition of the inner ear liquid and the hemolymph suggest the existence of regulatory mechanisms or transport processes for the maintenance of the concentration gradients. Such mechanisms are yet be discovered, but the colloidal material and accessory structures are good candidates for this function. Consequently, future studies should be oriented towards the discovery of structures such as ion pumps, junction gaps or specialised organs.

5.6. Conclusions

The bush-cricket inner ear is functionally and structurally less complex, yet smaller than those in mammals. For instance, the number of mechano-sensory cells is significantly lower in bush-crickets. Even so, the physical principles underlying hearing in mammals are the same for hearing in bush-crickets. The auditory organ is uncoiled and the tonotopic organization takes place in a relatively short distance (approximately one third of the length of the mammalian basilar membrane), and individual cap cells are visible on the surface of the tectorial membrane along the *crista acustica*. Such features provide an opportunity for experimental manipulation and, by the methods presented here, for the collection of high-quality data. The reduced number of auditory sensory neurons and the short length of the hearing organ in theory compromises frequency resolution in the bush-cricket ear. But certainly, these systems are not well understood and until the problem is rigorously approached, the phenomena of frequency resolution and sensitivity will remain elusive.

Measurement of auditory activity in bush-crickets was validated by presenting the assessment-of-suitability data for six species of bush-crickets with various level of cuticle pigmentation, and furthermore, analysing and presenting new data on travelling waves (velocity and wavelength) and tonotopy in one species of bush-cricket exhibiting the highest suitability criteria. This is the first time cuticle transparency has been studied in, and associated to, hearing research. Current work on katydid hearing in other labs usually focuses on one or two model species (e.g., *Mecopoda elongata*) and the methods utilised to access the inner ear are invasive and require major dissection, which might end in unrealistic results and inadequate interpretations. In this regard, this study is significant because it non-invasively measured six different Neotropical species from three different subfamilies, which also highlights potential new model species.

The presence of a liquid with features such as a specific ionic profile, incompressibility, and stable concentration and viscosity, contributes to the sensitivity, filtering, and tuning of the bush-cricket hearing organ. It is recognized that this study serves as a preliminary assessment both from the point of view of number of species investigated and the number of individuals per species. However, the obtained results herein offer the bases to further research in bush-crickets with analogous biochemical traits and mechanisms to the mammalian inner ear.

The potential impacts of this research are multiple, from expanding the knowledge of complex hearing processes which cannot be measured in the

mammalian inner ear, to opening new paths for research in hearing by integrating physical principles with novel information on sensory receptor function.

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Appendix A1.

A1.1. Acoustic analysis of male calling songs

It is shown here the acoustic analysis of the recorded songs of some of the species collected during the field work.

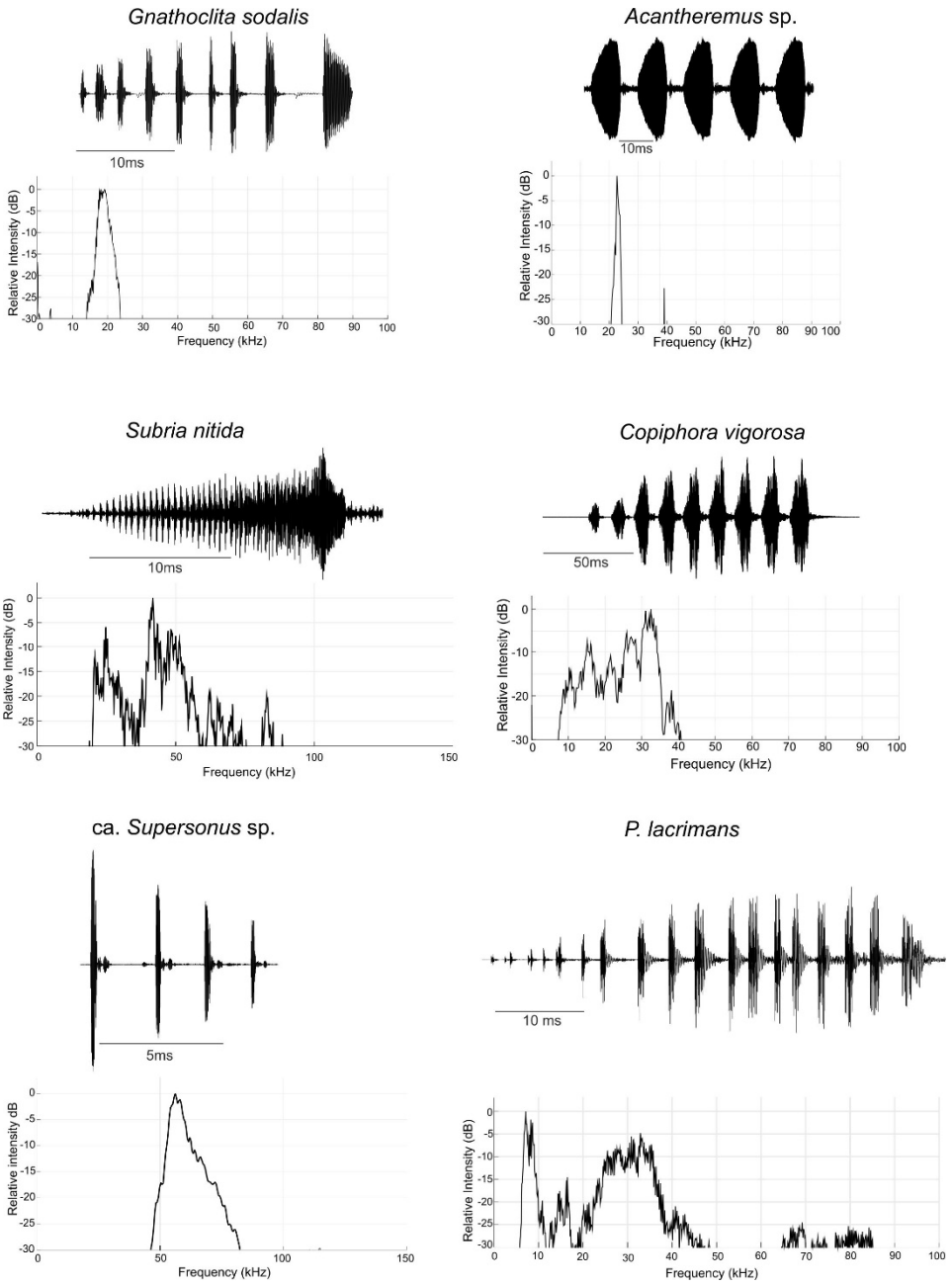

Figure A1.1. Song pulses in the time domain and power spectrums of some collected species.

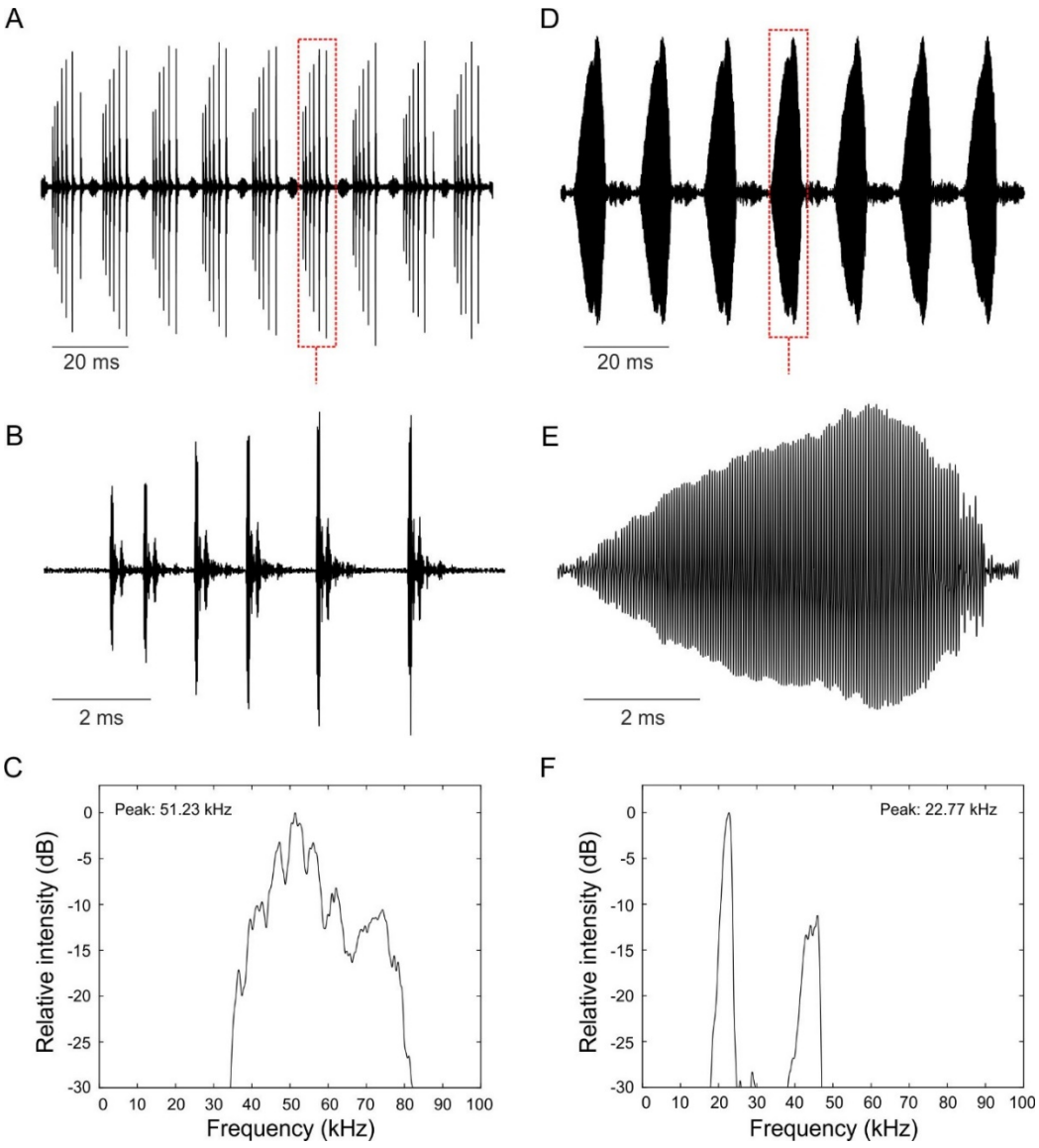

Figure A1.2. Acoustic analysis of the call of the two species exhibiting the highest cuticle light transmittance. A-C) *Phlugis poecila* and D-F) *Copiphora gorgonensis*. A and D) Typical presentation of the call. B and E) A single phonatome (closing stroke of the wings) in detail. C and F) Spectral analysis of the phonatome in B and E). Wide bandwidth of prevalent frequencies are apparent in the call of *P. poecila*. There is a higher tonal purity and harmonic content in the call of *C. gorgonensis*.

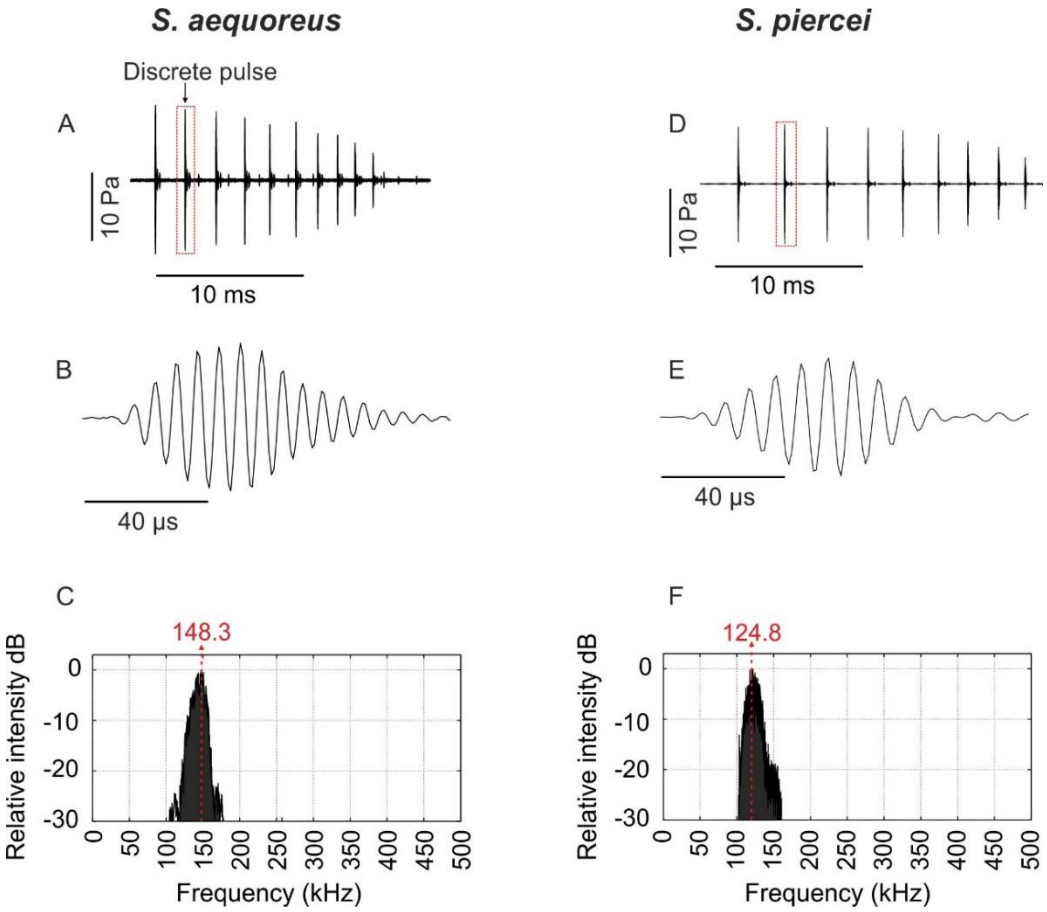

Figure A1.3. Calling song features of *Supersonus* spp. A) and D) Syllable or pulse train produced during a closing stroke of wing motion. B) and E) Wave form of a discrete pulse (red rectangle in A and D). C) and F) Power spectrum.